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Optimizing media and planktonic culture conditions for *in vitro* experimentation using free-living *Bradyrhizobium japonicum* USDA110

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by

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Optimizing media and planktonic culture conditions for *in vitro* experimentation using free-living *Bradyrhizobium japonicum* USDA110

Zoe Welch

Abstract

Bradyrhizobium japonicum USDA110 is an agronomically important bacterium with the ability to live as both an N_2-fixing, soybean symbiont and a free-living, heterotrophic cell. Free-living *Bradyrhizobium japonicum* USDA110 has been successfully cultured in lab settings since the 20th century, and has been used in a variety of planktonic growth-based studies using various culture media. Recent sequencing and annotation of the *Bradyrhizobium japonicum* USDA110 genome has enabled further "-omics" based advancements, thus increasing the attractiveness of envisioning *Bradyrhizobium japonicum* USDA110 as a model organism for *in vitro* work. Further, as numerous *in planta* studies have indicated that *Bradyrhizobium japonicum* USDA110 possesses an inherent sensitivity to a variety of environmental stressors, this tendency reifies a position held by some soil ecologists that rhizobia may be conceptualized of as indicator species for agricultural soil systems. As there is increasing interest in developing reliable, *in vitro*, high throughput (HTP) screening strategies for hazard assessment in environmental compartments, it is worthwhile to examine how testing with *Bradyrhizobium japonicum* USDA110 could be used towards this purpose for agricultural soil systems. However, in order to use it to test metal-based toxicants, it is necessary to optimize a reliable testing paradigm —inclusive of a *Bradyrhizobium japonicum* USDA110 growth medium that is simultaneously defined, environmentally-relevant, supportive of robust culture growth, and which has been designed

towards maintaining consistent exposure conditions over the duration of culture growth. Previously used *Bradyrhizobium japonicum* USDA110 growth media are problematic, largely due to considerations relating to undefined or chemically incompatible composition. This work customizes a better *Bradyrhizobium japonicum* USDA110 growth medium by using a stepwise approach of literature review (to create a comprehensive database of rhizobium growth medium recipes and construct a medium foundation), *in silico* modeling (to predict chemical speciation thus allowing for a "modeling out" of precipitates) for further medium design, and empirical testing (*Bradyrhizobium japonicum* USDA110 growth studies in varying, single-alteration medium formulations) to confirm the ability of the finalized medium design (ZY medium) to support optimal growth of *Bradyrhizobium japonicum* USDA110 within the tested constraints. I found that ZY supports robust *Bradyrhizobium japonicum* USDA110 population growth, and that microtiter culture conditions enabled high reproducibility of growth results as determined by specific growth rate and maximum population yield metrics calculated from hourly absorbance measurements. Optimized growth occurred with glycerol as the sole C source, nitrate as the sole N source, and with thiamine and biotin being absent from the medium. Further, I found evidence in support of P being a limiting nutrient for *Bradyrhizobium japonicum* USDA110. Preliminary Cu salt exposure studies show high reproducibility and possible dose-dependent effects, but also indicate that further system characterization is likely needed. This thesis work establishes a defined and environmentally-relevant USDA110 culture medium (ZY) and reliable testing methodology that facilitates planktonic growth and improved *in vitro* toxicity testing capacity.

1) Introduction

Nutrient cycling, primarily attributed to microorganisms including bacteria, is a high value ecosystem service (Costanza et al., 1997). In terrestrial systems, biological nitrogen fixation by prokaryotes (BNF) accounts for between 90-130 Tg of nitrogen fixed per year (Philippot and Germon, 2005; Galloway, 1998), though some authors estimate that up to 180 Tg nitrogen per year may be fixed via BNF in soil systems (Tiedje, 1988). These fluxes correspond to estimates of BNF in soil systems providing between 42-50% of the total nitrogen fixed globally per annum (Philippot and Germon, 2005; Tiedje, 1988). Philippot and Germon (2005) note that the fixed nitrogen resulting from bacteria, specifically from rhizobial symbioses (detailed below), accounts for the largest contribution of combined nitrogen in the terrestrial habitat. Therefore, nitrogen-fixing bacteria are important actors within the global nitrogen cycle, with special significance for terrestrial systems.

Bacterially-mediated nitrogen fixation contributes to soil fertility by supplying bioavailable nitrogen needed to support crop plant growth and reproduction. This phenomenon is fundamental to the functioning of agricultural systems, and constitutes a natural alternative to the use of fossil-fuel intensive, synthetic fertilizers. Bacteria that perform N_2-fixation only in association with a host plant are known as rhizobia, and have been shown to have higher rates of N_2-fixation (2-3 orders of magnitude difference) than those of asymbiotic nitrogen-fixing bacteria (Philippot and Germon, 2005). Therefore, it is estimated that the symbiotic association between rhizobia and their host plants can produce biologically-fixed nitrogen in the order of up to 350 kg N per ha per year, dependent on specifics of the rhizobia-plant associations (Philippot and Germon, 2005).

When in symbiosis within the roots of a soybean plant, a bacteroid, a specialized symbiotic form of a rhizobium cell, is able to naturally and efficiently convert diatomic nitrogen into the ammonia and ammonium needed for plant growth and reproduction. This process is known as symbiotic nitrogen fixation (SNF). SNF is catalyzed by the nitrogenase enzyme, whose synthesis and activity is highly dependent upon conditions of low oxygen (3 to 30 nM) (Fischer, 1994). Though *ex planta* nitrogenase activity of some rhizobia isolates has been demonstrated (Agarwal and Keister, 1983), it is largely accepted within the literature that for the majority of studied rhizobial species, initiation of a productive symbiosis resulting in high N_2-fixation levels and healthy bacteroids is contingent upon the presence of a host plant, or host-derived chemical signals (Vauclare et al., 2013; Pessi et al., 2007; Gage, 2004; Loh and Stacey, 2003; Fischer, 1994). Fischer (1994) notes that it is exceptional among rhizobia for a single species to be able to fix nitrogen both in pure culture and in symbiosis. A detailed overview of the central N_2-fixation genes in rhizobia (*nif*, *fix*, related subunits that compose nitrogenase enzyme, and accessory genes), the known and unknown/proposed functions of said genes, and the associated conditions known to regulate their expression is provided by Fischer (1994). SNF is energetically expensive – depending on the rhizobium species, SNF requires 16-42 molecules of ATP per every two molecules of ammonia produced (Haag et al., 2012; O'Brian, 1996). In return, the bacteroids are provided with the fixed carbon (photosynthate as dicarboxylic acids) they require to sustain their greatly-modified heterotrophic metabolism (Delmotte et al., 2010).

The symbiotic process resulting in effective SNF is complex, and is reliant on a number of successful rhizobium-plant cell signaling and signal transduction events (Loh and Stacey, 2003), as well as extensive genetic regulation on the part of the rhizobium to become

a viable, N_2-fixing bacteroid (Vauclare et al., 2013; Pessi et al., 2007). In the transition from free-living bacterium to bacteroid, a rhizobial cell will undergo extensive cell envelope remodeling (Robertson and Lyttleton, 1984; Whitehead and Day, 1997), alteration to carbon metabolism (Lodwig et al., 2003), upregulation of genes responsible for osmolyte biosynthesis and accumulation (Vauclare et al., 2013), and downregulation of genes required for cell growth and division, DNA repair, synthesis of ribosomal proteins, membrane protein biosynthesis, and branched-chain amino acid synthesis (Barnett et al., 2004; Becker et al., 2004; Capela et al., 2006; Karunakaran et al., 2009; Haag et al., 2012). Additionally, the nitrogen stress response regulatory system that operates under free-living conditions is disabled in bacteroids in order to safeguard the high production levels of ammonia (fixed nitrogen) needed by the host plant (Patriarca et al., 2002; Haag et al., 2012).

The rhizobium *Bradyrhizobium japonicum* USDA110 (hereafter referred to as USDA110) is an important model organism due to a number of considerations that are detailed in the following paragraphs. To delineate, USDA110 is important due to: 1) tractability for lab use, 2) SNF efficiency/agronomic importance, 3) economic importance via association with global commodity crop (soy), and 4) demonstrated environmental stressor sensitivities that may be used to advance predictive toxicology/risk assessment pursuits. Taken together, these considerations are used to optimize culture conditions for free-living USDA110 that may in turn prove suitable for future toxicity testing.

First, USDA110 is a culturable bacterium, with an aerobic, heterotrophic, slow-growing wild type that is capable of utilizing various C (glycerol, glucose, vanillate, arabinose, etc.) and N (nitrate, ammonium, etc.) sources (Sadowsky et al., 1983; Ito et al., 2006). Evidence from studies using auxotroph mutants suggests that the USDA110 wild type

is prototrophic (Kummer and Kuykendall, 1989; Ruan and Peters, 1992; Green and Emerich, 1997), and thus, like many Bradyrhizobia, does not require vitamin additions for culture growth (Quispel, 1974; Vincent, 1981), though an explicit statement ascribing prototrophy to the USDA 110 wild type was not found within the literature reviewed. Interestingly, it appears that different Bradyrhizobia species and strains may have markedly different reactions to vitamins such as biotin, though a thorough and comprehensive testing of the effect of each vitamin across all strains has not been performed (Quispel, 1974). Growth inhibition of some rhizobia species by biotin has been previously documented in the literature, though USDA110 was not explicitly noted (Quispel, 1974; Elkan and Kwik, 1968; Bunn et al., 1970). The USDA110 wild type has a sequenced and annotated genome (Kaneko et al., 2002) allowing for advanced molecular and "-omics"-based investigative techniques.

Second, USDA110 is a bacterium of great agronomic consequence due to its high N_2-fixation potential when in endosymbiotic association with a soybean host plant (Sadowsky and Graham, 1999), and its use as an agricultural inoculant (Plessner, 1993). When not existing as a nitrogen-fixing, microaerobic endosymbiont, USDA110 is also able to persist in soil systems in a free-living, aerobic, non N_2-fixing state for years without losing symbiotic effectiveness (Narozna et al., 2015; Moawad et al., 1988).

Fluxes of symbiotically fixed nitrogen attributed to human-induced cultivation of legumes and rice (rhizobial host plants) are estimated to range from 30-50 Tg nitrogen per year (Philippot and Germon, 2005; Galloway, 1998). In cases of subsistence farming, SNF is often relied upon to provide a key source of bioavailable N inputs to agricultural soils (Sanginga, 2003). In other contexts, enhancement of SNF via the intentional introduction of rhizobial bioinoculants is increasingly considered to be a means toward more sustainable

agricultural practices (Fox et al., 2007; Bohlool, 1992), and the growth of leguminous crops that support SNF is utilized as a strategy to reduce dependence on synthetic fertilizers (Fox et al., 2007).

Since the mid-20th century, farmers have increasingly relied on synthetic N fertilizers (via the Haber-Bosch process), instead of SNF, to help drive high agricultural yields (Bohlool, 1992; Socolow, 1999). The tools driving this "Green Revolution" have come under increasing scrutiny over time (Howarth, 2008; Bohlool, 1992; Pimentel et al., 1973). In the context of nitrogen, the large scale production of synthetic N fertilizers is energy intensive—requiring massive inputs of fossil fuels (Pimentel et al., 1973; Socolow, 1999). The Haber-Bosch process synthesizes ammonia from diatomic nitrogen using iron-based catalysts at high temperatures (400-600 C) and pressures (20-40 MPa) (Kitano et al., 2012). Maintenance of these extremes require high energy expenditure, and given the extent of commercial ammonia production (160 million tons per year), the Haber-Bosch process consumes more than 1% of the annual global power production (Kitano et al., 2012). As of 2011, annual synthetic fertilizer consumption in the United States was estimated to be 22 million tons (USDA ERS), equating to a value of over $80 billion (Good and Beatty, 2011).

However, it is often the case that not all of the synthetic fertilizer applied in-field is utilized by the crop plants, thereby creating a scenario of nitrogen over-application (Vitousek et al., 2009). Good and Beatty (2011) document the significant extent of nitrogen fertilizer over-application in developed countries, and the subsequent myriad negative environmental impacts resulting from nitrogen loss from croplands with artificially high nitrogen balances. Such impacts include nitrate pollution of drinking water sources (Galloway et al., 2008; Burkart and Stoner, 2007; Powlson et al., 2008), eutrophication of freshwater and marine

ecosystems with concomitant hypoxic/anoxic "dead zone" effects (US EPA: Hypoxia), and the emission of the potent greenhouse gas nitrous oxide (N_2O) (Denman et al., 2007). A biological maximum is close to being reached in developed countries' crop yields, and increasing fertilizer application is unlikely to produce significant additional gains (Good and Beatty, 2011; Fox et al., 2007).Good and Beatty (2011) argue that in order to sustainably provide sufficient crop yields for the increasing global population, best nutrient management practices (BNMPs) must be implemented, inclusive of legume bioinoculants and SNF with crop rotation/covercropping. The benefits of legume/SNF-based "tools" for advancing sustainable agriculture is supported by the work of Drinkwater et al. (1998) which shows that legume-based cropping (with demonstrated SNF activity) leads to reduced C and N losses from agricultural soils as compared to soils treated with synthetic fertilizers. A recent review by Mus et al. (2016) discusses the possibility of utilizing SNF to largely supplant synthetic fertilizer use, and notes the inherent challenges posed by attempting to bioengineer SNF functionality into crop plants outside of the legume family.

Herridge et al. (2002) notes that not all legumes respond similarly to bioinoculation. The nodulation response of some commonly-grown tropical legumes (green gram, soybean, black gram, groundnut, cowpea, chickpea, lentil, leucaena, pigeonpea, common bean) to bioinoculation can differ dependent on the host plant's nodulating characteristics for relative "promiscuity" (ability to form functional nodules with a range of rhizobial strains), as well as rhizobial populations, edaphic factors, and soil nitrate levels (Herridge et al., 2002; Thies et al., 1992). Herridge et al. (2002) discusses the large bioinoculation response of soybean (as measured by increased nodule counts and/or SNF efficiency) respective to the other aforementioned legumes as likely influenced by soybean's tendency for non-promiscuity,

and states that soybean is likely to benefit more from bioinoculation than many other legumes.

Third, soybean (*Glycine max*) is an important global commodity crop, acting as a primary protein and oilseed source for many populations and industries (IISD; USDA; FAO). Soybeans account for over 60% of global vegetable and animal meal production, and approximately 35% of the total harvested land area devoted to perennial and annual oilcrops (FAO Markets and Trade Division, Thoenes). Naturally, soybean plant health and productivity depends upon an effective symbiotic partnership with rhizobia. USDA110 is known for its N_2-fixation efficacy, and is commonly applied as an inoculum to soybean seeds prior to planting to ensure sufficient nodulation, N_2-fixation, and significant increases in harvest yields (Sadowsky and Graham, 1999). Phillips notes the work of Evans and collaborators (Albrecht et al., 1979; Schubert et al., 1978) which demonstrates that soybean plants grown with USDA110, instead of the related *Bradyrhizobium japonicum* USDA 31, possessed 31% greater N content and 24% more total dry matter when produced without combined N sources (Phillips, 1980). Additionally, a study investigating the comparative N_2-fixation efficiency of multiple rhizobia strains in soybean plants grown in Nigerian soil showed that USDA110 N_2-fixation efficiency exceeded that of both native rhizobia as well as another *Bradyrhizobium japonicum* strain (USDA138) sometimes used as a bioinoculant (Okereke and Onochie, 1996). USDA110 is also noted as being more symbiotically competitive (as measured by percent nodule occupancy in soybeans in mixed rhizobial inoculation experiments) in comparison to other select soybean-specific rhizobia (Kosslak et al., 1983). This comparative increased ability to outcompete other rhizobia for primary nodule occupancy has been demonstrated to persist within the in-field USDA110 population

over time (more than one planting season) even in the absence of field re-inoculation (Moawad et al., 1988).

Fourth, USDA110's sensitivity to various environmental stressors may enable it to be utilized to great value as an ostensible indicator species for soil N-cycle function within the field of predictive toxicology. Research shows that N_2-fixing soil bacteria may be especially sensitive to various agriculturally-relevant environmental stressors, including pesticides (Madhavi et al., 1993; Fabra et al., 1997; Arias and de Peretti, 1993; Fox et al., 2007; Kaszubiak, 1966; Gillberg, 1971), metals (Heckman et al., 1987; Broos et al., 2005; Ahmad et al., 2012), and engineered nanomaterials (ENMs) (Ge et al., 2012). Studies have found that rhizobia within the Bradyrhizobium genus are especially sensitive to metal oxide (MOx) ENMs (Ge et al., 2012), and that USDA110 bacteroids associated with soybean plants grown in MOx-ENM amended soil have reduced N_2-fixation capacity (Priester et al., 2012). Reduced Bradyrhizobia viability and N_2-fixation from environmental stressors may imply perturbation of soil N-cycling, soil fertility, and subsequent ecosystem service provision. This is possible due to the fact that N_2-fixation is regarded as a "narrow" process (Schimel and Schaeffer, 2012) whose enzymatic catalysts lack functional redundancy. This is especially important given the great diversity of microbes (Locey and Lennon, 2016), and thus the expectation that one type of microorganism – if stressed in the environment—could be readily replaced by a functionally similar organism without consequence to ecosystem functioning may be flawed. The utility of conceptualizing bacteria as ecological targets and subsequently using them to assess risk and safety in the rapidly changing arena of environmental toxicity testing has gained increasing attention (Holden et al., 2014). Prior work has noted the ability of rhizobia in particular to be utilized as important indicators of

soil health (Van Bruggen and Semenov, 2000; Visser and Parkinson, 1992), especially with application for agricultural soils due to noted high sensitivities to agrochemical inputs (Domsch et al., 1983). Precedent exists for using bacteria as indicator organisms for ecotoxicological screening in aquatic ecosystems (Park and Choi, 2008; Blaise, 1991). Building from this, recent research has attempted to adapt investigative methods and identify possible indicator organisms within soil ecosystems (Wessen and Hallin, 2011; Ritz et al., 2009). Anderson (2003) suggests that quantifying physiological measurements (such as C uptake and growth) of total microbial biomass per unit time is an important component in being able to assess soil health status as determined by eco-physiological indices. If USDA110 can be utilized *in vitro* to successfully and reliably assay for the effects of a given environmental stressor before it is introduced to a soil system, and this data can then be extrapolated to inform system-based models of N-cycling, then this could constitute an immense time and money saving benefit to the field of predictive toxicology.

As we consider how to optimize predictive hazard assessment for emerging contaminants, we must evaluate the efficacy and adaptability of our current toxicological toolkit (Dix et al., 2007). An important component of our toolkit for toxicity screening studies using bacteria is the culture media used, as its composition can have a large influence in determining toxicity outcomes (Jin et al., 2010). As noted by Bird and colleagues, even when rich growth media is diluted, its constituent components such as amino acids, proteins, and lipids can chemically interfere with metal species, thus leading to test results that may be subject to "serious misinterpretation" (Bird et al., 1985). A recent nanotoxicology paper by Bondarenko et al. (2013) lends further support to Bird's claim. Bondarenko et al. (2013) discusses the likelihood of metal ion toxicity (as measured by bacterial inhibition) being

reduced in rich media as opposed to mineral medium due to the presence of organic components. The authors argue that organic compounds complex with the metal ions, thereby inhibiting an ion's ability to induce toxic outcomes in bacteria (Bondarenko et al., 2013).

A focus is placed upon growth of heterotrophic, free-living USDA110 populations due to eventual implications for the host infection process that, when successful, results in symbiotic, N_2-fixing bacteroids. Growth of free-living USDA110 bacteria is important for maintaining viable USDA110 populations in agricultural soils over long timescales (Narozna et al., 2015). Perhaps more importantly though, growth of USDA110 ultimately determines if a successful, N_2-fixing symbiosis can be achieved. Though USDA110 rely upon flagella-driven motility to reach the exterior of a root hair cell, once inside the cell, progress is no longer driven by motility. In order for USDA110 to achieve a successful infection of the host, it must grow and divide up the length of the infection thread to reach the nodule primordium (Gage, 2004). Thus, the successful establishment of an N_2-fixing USDA110-soybean symbiosis is ultimately determined by the growth of free-living, heterotrophic USDA110.

Prior *in vitro* studies of USDA110 population growth and physiological response to introduced stressors and toxicants has utilized various culture media. The formulations of these media vary widely, and can fall anywhere on the spectrum between defined and minimal/nutrient-deplete to undefined and rich/nutrient-replete. There has been an increasing preference to study rhizobia, such as USDA110, in defined culture media that best represents conditions relevant to bacterial life in soil systems, namely, conditions of nutrient limitation. While generally an improvement over undefined, rich media for various testing applications, the defined and minimal media commonly used for in vitro culture is not theoretically

optimal for conducting planktonic toxicity testing. This is due to several reasons relating to changes in exposure conditions over time and background abiotic ROS generation, especially when used to test toxicity for metal-based materials.

First, many extant media rely on high concentrations of phosphate for buffering—a problematic situation due to phosphate's high tendency to bind divalent cations and precipitate these compounds out of solution (Watt, 1923). Such phenomenon may alter metal bioavailability and thus the exposure conditions of metal toxicity studies, and would impact the interpretation of toxicity effects as elicited by the solubilized fraction of a given metal. Second, HEPES, a common medium ingredient chosen either as a buffering agent or S source, has been demonstrated to complex with Cu^{2+} ions (Hegetschweiler and Saltman, 1986; Sokolowska and Bal, 2005), thus having the ability to impact evaluations of the role of solubilized versus particulate fractions in eliciting variable toxicity responses for MOx ENP exposures. Further, due to its formulation as a piperazine-containing buffer, HEPES has been demonstrated to form radicals, and has been subsequently cautioned against for use in studies considering redox processes (Grady et al., 1988; Kirsch et al., 1998). As many metals (Jomova et al., 2012) and ENPs (Karakoti et al., 2010; Dowding et al., 2013) are shown to be redox active, it is important that media are designed so as to minimize confounding effects that may result in misunderstanding of toxicity mechanisms or magnitudes. Components of a nutrient medium can have unforeseen effects upon a toxicant's form and availability (Halliwell, 2003; Bird et al., 1985). For example, Ruparelia et al. (2008) notes the increased dissolution of Cu^{2+} and Ag^{+} ions from their correspondent MOx ENPs when in the presence of media including peptone, yeast, salt and beef extract compared to DI water, though a definitive causative mechanism explaining this observation was not proven by the authors

(Ruparelia et al., 2008). Thus, it is imperative that attention is paid to the medium composition in order to anticipate, and in many cases prevent, interactions with tested toxicants that may impact mechanistic toxicity interpretations when considered in concert with other important design factors such as incubation temperature and culture aeration.

Further, it is important to design a medium that is representative of environmental conditions that a rhizobium may realistically encounter while living in the soil solution. Soil systems exhibit extreme habitat heterogeneity at all scales (Young et al., 2008; Young and Crawford, 2004); however, we can narrow this range of possibilities to focus on the microenvironments that facilitate bacterial population growth and survival. As noted by Young et al. (2008), the sub-millimeter scale is a key area of investigation for soil science. At this scale, microorganisms reside and interact, and they are typically well-adapted to conditions of nutrient limitation and desiccation stress (Young et al., 2008). For rhizobia like USDA110, osmotic stress is countered through osmolyte biosynthesis and accumulation (Sugawara et al., 2010). Hirsch (2010) provides an overview of rhizobial environmental stress adaptations, and details how rhizobial biofilms likely act as sources for planktonic cell dispersal. Rinaudi and Giordano (2010a) affirms the linked interdependency of planktonic and biofilm states for the survival of many rhizobial species in soils. USDA110 has been shown to exist in both biofilm and planktonic forms (Pérez-Giménez et al., 2009; Barbour et al., 1991). Studies have proposed that there is a reduced tendency toward biofilm formation and an increased tendency toward planktonic phenotype at the root tips, possibly due to low nutrient availability (Rudrappa et al., 2008a; Rinaudi and Giordano., 2010a). Thus, a defined minimal medium for culturing USDA 110 could well represent nutrient-depleted conditions in the soil environment; in addition, medium design could allow for controlled exposures of

metal or similar toxicants to the bacterial, for assessing their sensitivity to such toxicants during growth.

This thesis work seeks to establish a defined and environmentally-relevant USDA110 culture medium (ZY medium) and reliable testing methodology that facilitates planktonic growth and improved *in vitro* toxicity testing capacity.

2) Methods

2.1) Stepwise Medium Design Approach and Rationale

1. The approach to medium design began with a review of the composition of media previously used to study the growth of USDA110 *in vitro*, as well as other Bradyrhizobia and Rhizobia. Recipes for rich and undefined media—such as Modified Arabinose Gluconate (MAG), Yeast Extract Mannitol (YEM), and Peptone/Salts/Yeast extract (PSY) – containing chemically variable components such as yeast or peptone were excluded from consideration as a foundation for medium design, however, the nutrient concentrations from the media's mineral components were noted to inform considerations of possible physiological requirements specific to rhizobia. Defined Bradyrhiozobia mineral media compositions—such as Arabinose Gluconatem (AG) and those attributed as Tully's, Götz, Keyser and Munns, Vincent, and Bergerson-Norris—were then assessed for their inclusion of reagents deemed problematic due to their demonstrated abilities to interfere with or otherwise confound toxicity testing results. For example, HEPES was excluded due to its demonstrated ability to alter trace metal availability in aqueous *in vitro* culture (Zhao and Chasteen, 2006; Mash et al., 2003). Similarly, high phosphate concentrations were also deemed problematic

due to the high divalent cation complexation tendency of PO_4^{3+}. Thus, media recipes containing non-ideal conditions (undefined components like yeast and peptone; HEPES; high PO_4^{3+}) were excluded, but commonalities in mineral reagents and their corresponding concentrations across media were noted and used to establish a range of acceptable reagent concentrations used to construct a "foundation" medium recipe, hereafter referred to as proto-ZY.

2. Once proto-ZY was established, chemical speciation modeling using MINEQL+ was used to identify predicted precipitate species under conditions best approximating planktonic culture ("open" carbonate system, 30°C temperature, pH = 6.6). At this point, predicted formation of Fe precipitates and PO_4^{3+} precipitates was identified. Then, a stepwise approach was used to "model out" Fe precipitation by inclusion of citrate—a known Fe chelator utilizable by Bradyrhizobia as an exogenous siderophore (Plessner et al., 1993; Guerinot, 1990).

3. Once Fe precipitation had been successfully "modeled out," a similar stepwise approach was used to "model out" PO_4^{3+} precipitation with MINEQL+. PO_4^{3+} concentration was reduced in a stepwise fashion, an order of magnitude at a time, until no PO_4^{3+} precipitates were predicted to form. This zero precipitate medium formulation is hereafter referred to as ZY0.

4. ZY0 was then empirically tested for efficacy as a USDA110 culture medium. A series of stepwise empirical tests based upon alterations of nutrient concentration or source were then performed for the goal of optimizing USDA110 population growth and yield. Tested alterations to nutrient concentration (e.g. PO_4^{3+} concentrations of 50, 5, and 0.5 μM) reflected agriculturally-relevant value ranges as reported in the literature. Similarly, tested

alterations to nutrient form (e.g. NO_3^- versus NH_4^+) reflect agriculturally-relevant forms reported in the literature. Observations from these empirical growth studies (i.e. if an alteration supported, inhibited, or did not influence a higher maximum population yield or a higher specific growth rate) were then considered when finalizing the ZY medium formulation. In this manner, a balance was attempted to be achieved between minimizing precipitation and optimizing USDA110 population growth within environmentally-relevant nutrient boundaries.

2.2) Medium Design Criteria

2.2.1) Considerations for Buffering

As a significant number of considered growth media contained HEPES or (high concentrations of) PO_4^{3+} as a primary buffer, and as both were identified as likely problematic for growth and toxicity studies, alternative buffers were investigated. To address concerns relating to an appropriate alternative buffer to HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or phosphate, the Good's Buffer, MOPS (3-(N-morpholino) propanesulfonic acid) (Good et al., 1966) was chosen due to a number of considerations. MOPS provides excellent buffering capacity within biologically relevant pH ranges specific to USDA110 population growth and viability (Sadowsky et al., 1983). MOPS has a high aqueous solubility (Good et al., 1966; Zhao and Chasteen, 2006) and low lipid solubility (Yu et al., 1997; Ferguson et al., 1980). Also, MOPS has been shown to have weak to non-detectable chemical and biological complexation properties (Zhao and Chasteen, 2006; Ferguson et al., 1980) as assessed by a low tendency to influence and be influenced by salt effects, a low tendency to bind to biological substances, a high degree of chemical stability

over time, and an inability to act as analog inhibitor of any biochemical reaction (Ferguson et al., 1980). Work by Yu et al. (1997) identifies the tertiary amine structural feature of MOPS as being a crucial determinant of its inability to form complexes with metal ions. Mechanistic analysis of the process of metal complexation shows that the N-substituents (ethyl or larger) composing tertiary amine compounds, such as MOPS, influence the configuration of the compound such that the compounds are sterically inaccessible to solvated metal ions in the surrounding aqueous solution, and thus unable to participate in initial bond formation with said ions (Yu et al., 1997). Unlike other Good's buffers such as Dipso and HEPES, MOPS has not been demonstrated to bind divalent cations such as Cu^{2+} thus affecting the availability of trace metals to bacterial cultures *in vitro* (Zhao and Chasteen, 2006; Mash et al., 2003). Further, MOPS has been shown to have low to non-detectable measurable interactions with biological structures such as cell membranes (Ferguson et al., 1980). Ferguson et al. (1980) posits that this relative biological "inertness" may be due in part to the great polarity of the molecule, which largely prevents it from passing through biological membranes and accumulating within cells. Thus, MOPS does not provide a primary C source to USDA110, thus avoiding potential situations of diauxic culture growth that may complicate assessment of population growth.

2.2.2) Considerations for Soil Conditions and Bacterial Physiological Requirements

As soil microenvironments are very heterogeneous (Young et al., 2008; Young and Crawford, 2004) and possess high variability in the dissolved ion content of vadose zone water as determined by soil type and climate, it is extremely likely that USDA110 soil populations may experience a range of environmental conditions. However, as no single defined medium

can accurately replicate all soil microenvironment conditions, it is important to construct a medium informed by reported averages and ranges for various soil criteria (e.g. pH, Ionic Strength (IS), C:N ratio, P concentration), and, when possible, to reconcile these soil criteria values with those attributed to supporting the best possible growth and health of USDA110 populations. The pH of most productive agricultural soils typically ranges from 6.0 – 7.5 (CS Extension). Sadowsky et al. (1983) reports that slow-growing rhizobia such as USDA110 thrive within this range, and seem to prefer slightly acidic conditions—remaining viable at a pH of 4.5, though not at a pH of 9.0. IS can vary widely, ranging from 0.001 M to 1 M in some systems (Black and Campbell, 1982), though the commonly quoted average IS value for soil solutions is 0.03 M (Schofield and Taylor, 1955) , therefore a medium approximating this value would be ideal. To approximate the nutrient limitation that may exist in soil microenvironments, a relevant C:N ratio can be designed based on estimates of C-limited soil conditions (C:N ≈ 10) as identified by Mooshammer et al. (2014) (Figure 1). Phosphate concentrations in soil solutions can vary widely, ranging from 10^{-8} to 10^{-5} M dependent upon the microsphere (Beck and Munns, 1984; Reisenauer, 1966), and Bradyrhizobia have been demonstrated to have great variability in both P-uptake and utilization efficiency (Beck and Munns, 1984).

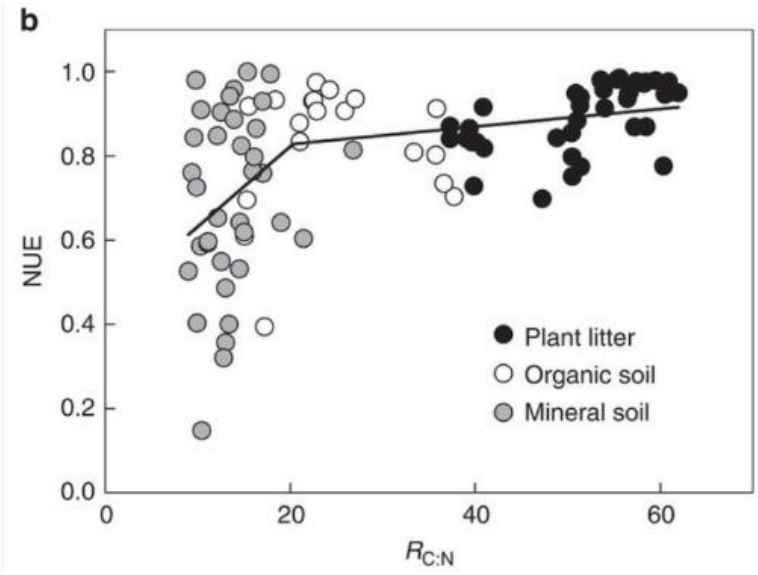

Figure 1. Relationship of C:N ratios to Nitrogen Use Efficiency. Graphic identifies the range of C:N values that translate to C-limitation for soil bacteria. From Mooshammer et al. (2014). Made available via a Creative Commons license viewable here: https://creativecommons.org/licenses/by/3.0/legalcode

2.3) *In Silico* Medium Modeling

Predictions of dissolved and precipitate fractions from medium reagents were calculated using MINEQL+ chemical speciation modeling software. To use the MINEQL+ software, a medium recipe's reagents must first be "broken down" to their ionic constituents. Molar concentrations of constituent ions were calculated according to each reagent, and then summed according to ionic identity (e.g. SO_4^{2-} ion concentrations from $MnSO_4$ and $ZnSO_4$ were combined) using Excel software. Within the MINEQL+ software interface, relevant ions corresponding to the medium composition were selected from the software database of chemical compounds, and the aforementioned "combined" ionic concentrations were assigned to their corresponding ionic species. Once ionic concentrations were input, environmental condition data (pH, temperature, nature of carbonate system) had to be specified before allowing the software to initiate running equilibrium modeling predictions.

The resultant chemical speciation modeling output reflects equilibrium conditions under an "open" system allowing for air exchange wherein CO_3^{2-} = -3.5. Further, modeling parameters included the additional environmental data reflective of biologically-appropriate USDA110 culture conditions (temperature = 30°C, medium pH = 6.6).

2.4) Sourcing, Validation, and Maintenance of Bacterial Cultures and Reagents

Pure *Bradyrhizobium japonicum* USDA110 stock was sourced from the US Department of Agriculture's Agricultural Research Service Culture Collection (USDA ARS NRRL). A single-colony isolate stock was created from this reconstituted culture, and was validated to be wild-type *Bradyrhizobium japonicum* USDA110 through 16S Sanger sequencing (primer sequences as specified in van Berkum and Fuhrmann (2000)) and subsequent comparison to the listed 16S entry derived from the USDA110 wild-type genome as described by Kaneko et al. (2002). Cultures were stored and maintained at -80°C in a 30/70 glycerol/LB stock contained within 1.5mL volume cryovials. All chemicals were reagent grade or better (Fisher Scientific, Hampton, NH; and Sigma Chemical, St. Louis, MO) and equaled or exceeded 95% purity.

2.5) Culture Growth

To perform planktonic growth studies, Modified Arabinose Gluconate (MAG) solid media (per liter of medium: 1.3g HEPES, 1.1g MES, 1.0g Yeast Extract, 1.0g L-arabinose, 1.0g D-Gluconic Acid sodium salt, 0.22g KH_2PO_4, 0.25g Na_2SO_4, 2.0 mL of 16g/100mL NH_4Cl stock solution, 1.0mL of 0.67g/100mL $FeCl_3$ stock solution, 1.0mL of 1.5g/100mL $CaCl_2$ dihydrate stock solution, and 1.0mL of 18g/100mL $MgSO_4$ heptahydrate stock

solution; bring volume to 1L using DI water; pH to 6.6 with KOH and add 18g Bacto-Agar per liter if making solid media; autoclave 20-30 min at 120°C) plates were struck from a -80°C USDA110 stock and were allowed 6 days of outgrowth (dark, 30°C) to accumulate sufficient colony biomass. USDA110 colony morphology was always noted to be consistent as small, white, smooth and convex. Axenic inoculum for planktonic studies was created by using a sterile loop to gather sufficient biomass into 4 mL of ZY medium (final ZY medium with alteration dependent on the empirical nutrient test being examined) which was then washed by vortexing for 45 seconds, and centrifuging for 10 minutes at 6500 rpm. The supernatant was then decanted, and the pellet was resuspended in 5 mL of fresh ZY medium. 200 µL of this concentrated inoculum was then removed and, alongside 200 µL of sterile ZY, both samples were measured for absorbance (Optical Density at λ=600, abbreviated as OD_{600}) using an automated microtiter plate reader (Biotek Synergy2; Winooski, VT)). The reading for the sterile ZY was subtracted from that of the concentrated inoculum, and then the inoculum was diluted so as to have a starting $OD_{600}$of 0.16. When a midiculture format was used, the concentrated inoculum volume (5 mL) and means of measuring concentrated inoculum absorbance (200 µL in a well in a 96-well plate in the Biotek Synergy2 plate reader) remained the same, but an increased amount of biomass was included in the concentrated inoculum, thus producing a higher absorbance that could then be diluted out to 2X working concentrations over larger volumes.

A microtiter 96-well culture format was employed for the bulk of this research due to consideration of benefits, namely cost-effectiveness, associated with high throughput (HTP) and high content (HC) screening strategies (Major, 1998). Overall though, this research used two different culture formats to produce growth curves: a microtiter format utilizing clear

polystyrene 96-well plates read with a Biotek Cytation3 instrument wherein culture volumes were 200 µL, and a midiculture format utilizing 15 mL borosilicate culture tubes coupled with %Transmission readings from a manual spectrophotometer (Spectronic 20+, Spectronic Instruments) wherein culture volumes were 10 mL. The midiculture format was only employed for one experiment—when investigating if culture format influenced the growth of USDA110 populations (see Methods section 2.7.6.2, and Results section 3.4.6.2).

Percent transmission readings from the manual spectrophotometer were converted to absorbance using the formula:

$$A = 2 - \log_{10} \%T$$

When samples were loaded into the microtiter plate before a microtiter format growth study was to begin, 100 µL of the OD_{600}0.16 inoculum was combined in well with 100 µL of fresh media, thus creating a starting OD_{600} of 0.08. This method was scaled up for midiculture growth studies (5 mL of OD_{600}0.16 inoculum combined in tube with 5 mL of fresh media), and also resulted in an initial starting OD_{600} of 0.08.

Uninoculated controls were always run alongside experimental treatments in order to confirm successful aseptic technique and provide a background optical control for use in data processing. Replicate number in all experimental and control treatments was always greater than or equal to 3. Population growth metrics (specific growth rate and maximum yield) were calculated, and analyses for statistical significance were performed using Excel and the JMP Pro11 statistical program (see Section 3.3 for further details). All microtiter runs were conducted in a Biotek Synergy 3 microplate instrument under 30°C shaking (200 rpm) incubation using clear, 96-well polystyrene plates with fitted (but not airtight) lids (Costar), and growth was allowed to proceed for 95 hours in the dark with absorbance readings taken

every hour. All midiculture runs were conducted in a shaking incubator (30°C, 200 rpm) under dark conditions with absorbance readings taken every 1-4 hours contingent on the phase of culture growth.

2.6) Growth Curve Calculation and Data Analysis

Data collection and export were performed using Gen5 Microplate Reader and Imager Software (Biotek) and 2013 Microsoft Office Excel software. Data consolidation, graphing, and growth metric calculations were performed using 2013 Microsoft Excel and custom Python script Spekit v0.2.2 (https://bitbucket.org/swafford/spekit). Full source code can also be viewed in Appendix B. Spekit v0.2.2 was created for this work in collaboration with A. Swafford. Spekit v0.2.2 is able to mathematically identify the data range corresponding to the approximate exponential phase in a bacterial growth curve, and then allows the user to "fine-tune" the selection of exponential phase start and end points by exploring the associated R^2 rankings and line-of-best-fit graphic overlays of these start-end pairs. To ensure that variations in background optics were accounted for, an averaged value calculated from concurrently run, paired abiotic treatment controls was subtracted from biotic growth data. Tests for statistical analysis were performed using both Excel software and JMP Pro 11 software. 1-way ANOVAs and post-hoc statistical analyses using Tukey's Honesty Significant Difference test ($\alpha = 0.05$) were performed to evaluate significance of the growth curve metrics: specific growth rate (SGR in h^{-1}) and maximum yield at a wavelength of 600 nanometers (max OD_{600}).

2.7) Empirical Testing of Population Growth Response to Nutrient Alteration

2.7.1) Carbon Source

Various C sources can support USDA110 population growth (Green et al. 1998; Green and Emerich, 1997; Sadowsky et al., 1983; Kuykendall and Elkan, 1976) however, there is a lack of complete understanding regarding how different C sources may comparatively alter population growth characteristics when in minimal, defined media. Therefore, investigation into the possibility that USDA110 population growth characteristics may vary due to the form of C provided was performed. The effect of varying C source upon planktonic USDA110 growth in liquid ZY media was tested. Glycerol and L-arabinose were chosen as C sources due to their prevalence of use in Bradyrhizobium culture media (Hohle et al., 2011; Green et al., 1998; Green and Emerich, 1997; Frustaci et al., 1991; Sadowsky et al., 1983; Kuykendall and Elkan, 1976) and reported ability to support robust population growth from both published (Kuykendall, 2005) and unpublished sources (personal communication with Dr. Michael Sadowsky). Further, glycerol has been noted as the most universally utilized carbon source amongst rhizobia (Stowers, 1985). Though mannitol has been utilized to grow Bradyrhizobia in various previous studies, provision of mannitol has been attributed to causing large variability in culture growth (Stowers, 1985), and selection of mutants divergent from the wild type (personal communication with Dr. Patrick Elia, USDA). Either glycerol or L-arabinose as sole C sources were supplied in equal molar concentrations. All other variables (N source and concentration, P source and concentration, pH 6.6, microtiter format, starting OD_{600}= 0.08) were kept constant. C:N ratio was maintained at 11. Phosphate was supplied in a concentration of 50µM. The N-source was nitrate at a concentration of 5.9 mM, and no vitamins were added. Each experimental group had technical replication of $n \geq 3$.

2.7.2) Carbon Source + Vitamins

As 1) the literature indicated that response to vitamin additions may vary widely across members of the *Bradyrhizobium* genus, 2) as relevant growth media varied in their inclusion of biotin and thiamine, and 3) no definitive indication of USDA110 prototrophy was stated in the literature reviewed, an empirical testing of USDA110's growth response to biotin and thiamine inclusion under the provision of either of two different C sources was performed. The effect of an additional vitamin solution (comprised of biotin and thiamine) upon planktonic USDA110 growth in liquid ZY media was tested using either glycerol or L-arabinose as the sole C source. All other variables (C:N ratio = 11, N source and concentration, P source and concentration, pH 6.6, microtiter format, starting OD_{600}= 0.08) were kept constant. Phosphate was supplied in a concentration of 50µM, and 5.9 mM nitrate constituted the N-source. Each experimental group had technical replication of $n \geq 3$.

2.7.3) Carbon Concentration

The effect of varying C concentration upon planktonic USDA110 growth in liquid ZY media was tested. Glycerol and glucose were compared; glycerol due to its prevalence in Bradyrhizobia growth media (Hohle et al., 2011; Green et al., 1998; Green and Emerich, 1997; Frustaci et al., 1991; Sadowsky et al., 1983; Kuykendall and Elkan, 1976) and recommendation for use by Dr. Michael Sadowsky (personal communication), and glucose due to its demonstrated ability to support rhizobial growth (Kuykendall and Elkan, 1976; Martinez-De Drets and Arias, 1972) and its noted ability to be easily catabolically utilized via numerous metabolic pathways by many bacterial species (Gottschalk, 2012). Either

glycerol or glucose were supplied in two different C concentrations, equating to a C:N ratio equal to either 11 or 22 M. 11M well represents C-limiting conditions and 22M represents non-limiting conditions (Mooshammer et al., 2014). All other variables (N source and concentration, P source and concentration, pH 6.6, microtiter format, starting OD_{600}= 0.08) were kept constant. Phosphate was supplied in a concentration of 50 µM. The N-source was nitrate in a 5.9 mM concentration, and no vitamins were added. Each experimental group had technical replication of n≥3.

2.7.4) Nitrogen Source

Different inorganic N sources have been used in rhizobial growth media without a clear acknowledgment of which may produce better growth results for individual species and strains (Vincent, 1981), as understood by higher growth rates and/or higher population yield. For instance, Vincent et al. (1970) does not specify the form of N to include in the liquid growth medium recipe, simply listing "N-source 0.8g/L". Therefore, the effect of varying N source upon planktonic USDA110 growth in liquid ZY media was tested in order to generate strain-specific results. Both nitrate (Bergersen, 1961; Ayanaba et al., 1983) and ammonium (Green and Emerich, 1997; Götz et al., 1982; Cole and Elkan, 1973) have been used as N sources in rhizobial culture media, and both are the primary N forms in most soils (Maynard and Kalra, 1993). Either nitrate or ammonium was supplied in equal concentrations (5.9 mM), and the counter ions (potassium and chloride) were balanced accordingly. All other variables (C source and concentration, P source and concentration, pH 6.6, microtiter format, starting OD_{600} = 0.08) were kept constant. C:N ratio equaled 11M; C was provided as glycerol. No vitamins were added. Phosphate was supplied in a concentration of 0.5 µM as

this test preceded the testing of variable phosphate concentrations, and the resultant increase of ZY medium phosphate levels. Each experimental group had technical replication of $n \geq 3$.

2.7.5) Phosphate Concentration

As USDA110 planktonic populations have been typically grown in phosphate-replete conditions (Beck and Munns, 1984), there is merit in examining how bacterial population growth may differ under less abundant phosphate conditions which reflect concentrations often found in agricultural soils (Beck and Munns, 1984; Cassman et al., 1981a; Cassman et al., 1981b). The effect of phosphate concentration at 3 different orders of magnitude (50, 5, and 0.5 µM) representing relevant phosphate ranges in agricultural soil solutions (Beck and Munns, 1984) upon planktonic USDA110 growth in liquid ZY media was tested. The highest phosphate concentration tested (50 µM) was at least an order or magnitude less than that found in many rhizobial growth media (Bergersen, 1961), thereby mitigating complexation and precipitation effects. The range of phosphate concentrations tested had previously been shown to support USDA110 population growth while remaining relevant to average phosphate ranges in typical agricultural soils (Beck and Munns, 1984). All other variables (N source and concentration, C source and concentration, pH 6.6, microtiter format, starting OD_{600}= 0.08) were kept constant. The N-source was nitrate in a 5.9 mM concentration, the C source was glycerol, a C:N ratio of 11M was maintained, and no vitamins were added. Each experimental group had technical replication of $n \geq 3$.

2.7.6) Finalized ZY Medium: Tests for Reproducibility & Format

2.7.6.1) Reproducibility

The finalized ZY medium formulation was tested for reproducibility of planktonic USDA110 population growth. The finalized ZY medium formulation was arrived at following a step-wise, multi-method process that resulted in a medium that simultaneously showed no visualizable precipitation and produced highest specific growth rate and maximum population yield values within the parameters tested. To produce the USDA110 biomass inocula needed for planktonic studies, two different solid MAG plates were struck from the same -80°C single colony isolate stock and were incubated under identical conditions (dark, 30 °C) for 6 days on two separate dates. Then, these inocula were used to grow planktonic USDA110 populations. All experimental conditions (medium composition wherein C source was glycerol, the C:N ratio was maintained at 11M, N source was nitrate at a 5.9 mM concentration, phosphate concentration was 50 µM, no vitamins added, pH == 6.6, microtiter culture format) were held constant between the two experimental dates. All starting OD_{600} values were standardized to 0.08. Both experimental dates had technical replication wherein n=5 (wells). Tests for reproducibility were only undertaken for the microtiter culture format, not for the midiculture format.

2.7.6.2) Format (microtiter versus midiculture)

The finalized liquid ZY medium formulation was tested to see if planktonic USDA110 growth over time varied dependent on the culture format used. The rationale was to establish a means of comparison between a low-throughput, high volume culture format and a high-throughput, low-volume format, and assess the degree with which the formats might be interchangeable in culture work. My research used finalized ZY medium to compare two formats: 96-well, clear polystyrene microtiter plates with a working culture

volume of 200 µL, and borosilicate midiculture tubes with a working culture volume of 10 mL. Minus culture format, all experimental conditions (medium composition wherein C source was glycerol, C:N ratio was maintained at 11M, N source was nitrate at a 5.9 mM concentration, phosphate concentration was 50 µM, no vitamins added, pH == 6.6, microtiter culture format) were held constant. All starting OD_{600} values were standardized to 0.08. Each experimental group had technical replication of $n \geq 3$.

2.8)-*In Vitro* Test for Citrate Utilization using Simmons Citrate Solid Medium

USDA110 was previously described as unable to utilize citrate as primary C source (Sadowsky et al., 1983), however, a colorimetric citrate test was conducted to ensure that this characteristic was possessed by my USDA110 single colony isolate stock. Testing for primary C source citrate utilization using Simmons Citrate Medium was conducted using *Pseudomonas aeruginosa* PG201(originally sourced from Urs Ochsner, University of Colorado) as a positive control (is able to utilize citrate as a primary C source). To test, a small amount of archived PG201 bacteria (maintained at -80°C in 70% LB plus 30% glycerol v/v) was aseptically transferred onto the surface of a sterile Simmons Citrate solid medium plate, and an identical method was used to inoculate a Simmons Citrate solid medium plate sourced from the same batch of solid plates using my USDA110 single colony isolate stock (maintained at identical -80°C archival conditions as PG201). The plates were then inoculated using a loop flame-sterilized between uses, and allowed outgrowth at 30°C (dark). Plates were monitored daily for colorimetric change.

2.9) Preliminary Testing of Cu Salt Exposure

To test for applicability of finalized ZY medium in metal toxicity testing, preliminary exposure testing of Cu salts to USDA110 populations grown to stationary phase was performed. This testing consisted of 10 biotic treatments—a negative control containing USDA110 inocula but no additional Cu, and a 3^2 full factorial design (2 factors: Cu form and Cu concentration; 3 levels per each factor: 3 Cu salts ($CuSO_4$, $CuCl_2$, Cu Acetate) and 3 elemental Cu concentrations (0.26, 2.6, 26 ppm). Thus, the treatments were as follows: biotic control at 0 ppm elemental C; $CuSO_4$ at 0.26, 2.6, and 26 ppm elemental Cu; $CuCl_2$ at 0.26, 2.6, and 26ppm elemental Cu; Cu Acetate at 0.26, 2.6, and 26 ppm elemental Cu. Exposure testing was performed only in a 96-well microtiter format. Finalized ZY medium was used, thus the C source was glycerol, the C:N ratio was maintained at 11M, the N source was nitrate at a 5.9 mM concentration, the phosphate concentration was 50 µM, no vitamins were added, and the pH was 6.6. Cu salts were all reagent grade or higher (Fisher Scientific, Hampton, NH; and Sigma Chemical, St. Louis, MO). Exposures were created by introducing 100 µL of a 2X concentrated inocula (USDA110 in ZY medium) to the microplate wells in a manner identical to that previously described. Then, 100 µL of a 2X concentrated Cu solution (given Cu salt at double the desired exposure concentration measured as elemental Cu in ZY medium) was added to the well. This resulted in the desired 1X concentration of USDA110 culture (OD_{600} = 0.08 in biotic control) and desired 1X concentration of elemental Cu. To note, though each well corresponding to a biotic treatment received the same amount of 2X inocula (OD_{600} = 0.16) to be ostensibly diluted by half to a starting OD_{600} of 0.08, due to optical interferences from the Cu salts, the starting OD_{600} values of all biotic wells were not identical across treatments. Each experimental group had technical replication of n=3. Corresponding abiotic controls (no inocula added) were included (n=3 technical replicates

per treatment) for each treatment in order to correct for Cu-related optical effects in the biotic absorbance data Additionally, it should be noted that as finalized ZY medium contains Cu in concentrations demonstrated to be biologically beneficial (Table 1), usage of terminology such as “added Cu” or “additional Cu” refers to Cu that has been provided on top of this Cu baseline, thus creating exposure scenarios resulting in possible, Cu-caused, toxicity outcomes.

3) Results

3.1) Stepwise Medium Formulation

ZY medium composition was first informed by compiling the recipes of existing Rhizobium-specific media recipes and cross-referencing them with extant literature detailing the macro and micronutrient requirements of Bradyrhizobia and Rhizobia in order to assess basic nutrient ranges, as well as commonalities and differences in growth media reagents. Once determined, this preliminary composition (proto-ZY) was cross-referenced with calculated mineral solubilities (Table 1) and then assessed for precipitate formation using MINEQL+ chemical speciation modeling software. Using this software, a zero-precipitate media formulation (ZY0) was determined by adjusting the concentration and/or form of phosphate-based reagents, Fe-based and associated reagents, and primary buffering reagents. The ability of this resulting medium formulation (ZY0) to grow *Bradyrhizobium japonicum* USDA110 was then tested empirically, and a subsequent adjustment to the concentration of phosphate-based reagents was made due to empirical evidence demonstrating that increased phosphate supports a higher SGR and maximum yield in USDA110 cultures (Table 2). After

empirically and systematically testing the effect of alterations to macronutrients composing the medium, a finalized version of ZY was achieved—this finalized ZY only differs from ZY0 by containing an increased concentration of phosphate (50 µM). Subsequent predictive chemical speciation modeling with MINEQL+ shows that under the conditions previously mentioned ("open" carbonate system, 30°C temperature, pH = 6.6), the only precipitate species predicted to occur due to abiotic factors in finalized ZY is $MnHPO_4$ at a concentration of 3.73 µM (Table 2; Appendix A, Table A). Regarding this final ZY composition, no precipitates can be seen in this medium with the naked eye, even months after creation. Empirical testing of ZY medium demonstrates satisfactory growth of USDA110 (Figure 2) using glycerol as the primary carbon source, though other carbon source substitutions may theoretically be made.

Element	*Reported Biologically Relevant Conc. as Rhizobia Nutrient*	*Reference*	*Calculated Solubility*	*ZY Medium Conc.*
P	50 nM - 1µM*	Graham (1992); Cassman et al. (1981a &b); Beck & Munns (1984 & 1985)	nc	50 µM
Ca	25-50 µM 2.5 µM >1 µM	Graham (1992); Vincent (1962); Keyser & Munns (1979) Bardin & Finan (1998) Karr & Emerich (2000)	21 µM	21 µM
Mg	0.5 mM 2.5 µM	Karr & Emerich (2000) Bardin & Finan (1998)	1.6 mM	2 mM
Fe	0.1 µM to 10 µM 0.5 µM to 5 µM 0.3 µM to 20 µM	Guerinot and Yi (1994) Guerinot et al. (1990) Jaggavarapu & O'Brian (2014)	4.41×10^{-10} M	20 µM
Zn	0.4 µM	Ayanaba et al.(1983)	7.9 µM	0.4 µM
Mn	0.4 µM to 50 µM	Hohle & O'Brian (2012)	nc	4 µM

*Table 1. Comparison of select, important rhizobia nutrients thought to pose difficulties regarding precipitation against solubility constraints and reported nutrient concentrations relevant to Bradyrhizobia physiology from other media. Elemental concentrations corresponding to final ZY medium formulation are included in far-right column for purposes of comparison. * indicates minimum required concentrations. nc = not calculated.*

REAGENT NAME	REAGENT CONC. (M)	
	ZYO	Finalized ZY
NA2HPO4	5.00E-07	5.00E-05
CACL2*2H2O	0.000021	0.000021
NACL	0.003425501	0.003425501
NA2MOO4*2H2O	2.93957E-05	2.93957E-05
MGSO4	0.002	0.002
KNO3	0.00593453	0.00593453
ZNSO4*7H2O	0.0000004	0.0000004
MNSO4*H2O	0.000004	0.000004
CUSO4	1.5726E-06	1.5726E-06
NA2B4O7*10H2O	4.74354E-07	4.74354E-07
COSO4*7H2O	8.89354E-07	8.89354E-07
DISODIUM EDTA DIHYDRATE	7.43592E-06	7.43592E-06
FESO4*7H2O	0.00002	0.00002
SODIUM CITRATE DIHYDRATE (NA3C6H5O7*2H2O)	0.001937534	0.001937534
GLYCEROL	0.021717885	0.021717885
MOPS	0.04	0.04
PREDICTED SOLID PRECIPITATE NAME	None	$MnHPO_4$
PREDICTED SOLID PRECIPITATE CONC.		0.00000373

Table 2. Differences in reagent concentrations between the zero-precipitate medium formulation (ZY0 and the finalized ZY medium composition.

3.2) Considerations for Soil Conditions and Physiological Requirements of Rhizobia

The finalized ZY medium composition well represents hypothetical conditions experienced by USDA110 free-living populations within the soil solution of the upper vadose zone. Considering the highly heterogeneous nature of the soil microenvironment, and the high variability in the dissolved ion content of vadose zone water as determined by soil type and climate, it is extremely likely that USDA110 populations experience conditions represented by ZY medium with respect to pH, ionic strength, and nutrient availability. ZY medium has been formulated to have a pH of 6.6, which is both relatable to the pH of many agricultural soils (CS Extension), and also falls within the allowable range conducive to USDA110 viability and growth (Sadowsky et al., 1983). ZY has a calculated Ionic Strength

(IS) of 0.033 M (Table 4), which relates well to the commonly quoted average value for soil solutions: 0.03 M (Schofield and Taylor, 1955). Relatedly, IS can vary widely, ranging from 0.001 M to 1 M in some systems, and the IS of ZY medium fits within these bracketed values (Black and Campbell, 1982). ZY medium's IS of 0.033 M (Appendix A, Table B) is comparable to the IS of other minimal bacterial culture media that have been used to great success in the field of environmental toxicity testing (e.g. Modified Minimal Davis medium has an IS = 0.057 M) (Horst et al., 2012). ZY has been formulated to have macro and micronutrient concentrations sufficient to support USDA110 nutritional requirements, but minimal enough so as to prevent creation of rich/nutrient-replete conditions (Table 1). Further, ZY provides a molar C:N ratio of 11. As evidenced in the provided population growth curves (Figure 2; Control treatments in Figures 6, 7, and 8), this ratio of C:N is high enough to support sufficient population growth yield, but also represents a reasonable estimate of C-limited soil conditions as identified by Mooshammer et al. (2014) (Figure 1). Additionally, ZY contains a phosphate concentration of 50 µM, which corresponds to a concentration commonly found in soil solutions and fertile soils (Beck and Munns, 1984; Reisenauer, 1966), and thus supports robust growth of USDA110 while remaining relevant to agricultural systems.

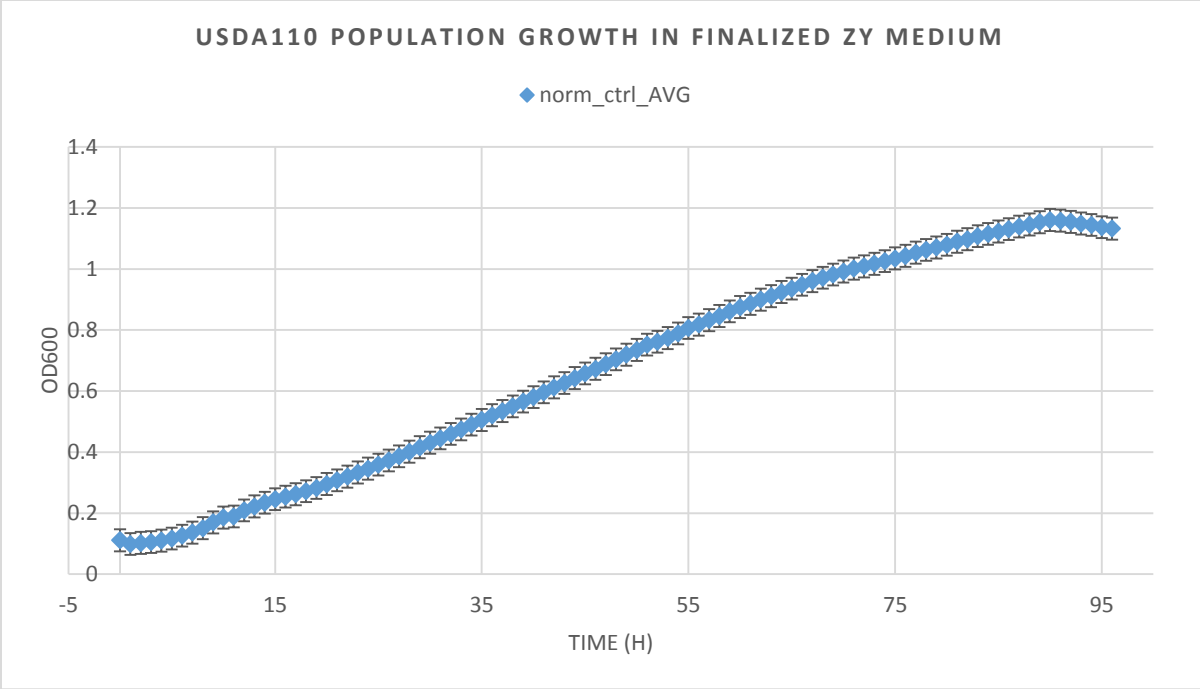

Figure 2. USDA110 population growth curve showing control treatment (n=3) averaged values with standard error bars.

3.3) Finalized ZY Medium Recipe

Finalized ZY Medium reflects a synthesis of results from literature review, *in silico* modeling, and empirical *in vitro* testing, and consists of a combination of 3 separate solutions: a base solution, a metals concentrate solution, and a phosphate concentrate solution. Their respective compositions are detailed as follows:

***Base solution*:** $FeSO_4*7H_2O$ (0.0056 g/L), Sodium citrate dihydrate (0.5698 g/L), NaCl (0.200 g/L), $Na_2MoO_4*2H_2O$ (0.0071 g/L), $MgSO_4$ (0.2407 g/L), KNO_3 (0.6 g/L), and MOPS (8.3705 g/L).

Metals concentrate solution: $CaCl_2*2H_2O$ (0.3087 g/100 mL), $ZnSO_4*7H_2O$ (0.0115 g/100 mL), $MnSO_4*H_2O$ (0.0676 g/100 mL), $CuSO_4$ (0.0251 g/100 mL), $Na_2B_4O_7*10H_2O$ (0.0181

g/100 mL), $CoSO_4*7H_2O$ (0.0249 g/100 mL), disodium EDTA dehydrate (0.2767 g/100 mL).

***Phosphate concentrate solution*:** Na_2HPO_4 (0.7098 g/100 mL).

All solutions are made using Nanopure water, and are filter sterilized (0.22 µm) before combining. 998 mL of Nanopure water is added to base reagents to make the base solution. Glycerol (2 g/L) is then added to the base solution. 1 mL of metals concentrate solution and 1 mL of phosphate concentrate solution are then added, resulting in 1L of 1X ZY medium. pH to 6.6 using KOH. The complete ZY solution is then filter sterilized using a 0.22 µm filter. The finished solution is then stored in the dark at room temperature.

3.4) Empirical Testing of Population Growth Response to Nutrient Alteration

3.4.1) Carbon Source Affects Growth

Results of testing for growth effects caused by differences in carbon source showed that both the average SGR (p=0.0081) and average max OD_{600} (p=<0.0001) were significantly different if glycerol was used as a sole C-source instead of L-arabinose. Glycerol as a sole C source produced higher average SGR (0.075 h^{-1}) as well as higher average max OD_{600} (1.2) than L-arabinose as a sole C-source (avg SGR 0.070 h^{-1}; avg max OD_{600} 0.75). At this time, a definitive and comprehensive explanation underlying the differences in observed growth results between glycerol and L-arabinose is not possible because slow-growing rhizobia, such as USDA110, have been demonstrated to utilize various metabolic pathways for C catabolism, each featuring various molecular actors and

processes of energy transfer not yet fully characterized (Stowers, 1985; Appendix A, Figure A).

3.4.2) Vitamin Effect Contingent upon Carbon Source Provided

3.4.2.1) *Effect of Vitamin Solution with Glycerol as Sole Carbon Source*

When glycerol was provided as the sole C source, there was a significant difference between both the average SGR ($p=0.0215$) and the average max OD_{600} ($p=0.0019$) of the culture grown in the presence of the vitamin solution (avg SGR 0.071 h^{-1}, avg max OD_{600} 1.13) versus the culture with no added vitamins (avg SGR 0.075 h^{-1}; avg max OD_{600} 1.2). Interestingly, the addition of biotin and thiamine seems to produce an inhibitory effect to USDA110 grown in the presence of glycerol. Growth inhibition of some rhizobia species by biotin has been previously documented in the literature, though USDA110 was not explicitly noted (Quispel, 1974; Elkan and Kwik, 1968; Bunn et al., 1970).

3.4.2.2) *Effect of Vitamin Solution with L-Arabinose as Sole Carbon Source*

When L-arabinose was provided as the sole C source, there was no significant difference in either the average SGR ($p=0.6899$) or the average max OD_{600} ($p= 0.8633$) when comparing the presence (avg SGR 0.0698 C; avg max OD_{600} 0.753) versus absence (avg SGR 0.0701 h^{-1}; avg max OD_{600} 0.753) of the biotin and thiamine vitamin solution. Neither an inhibitory nor stimulatory effect of the vitamin solution upon USDA110 population growth was observed when L-arabinose was provided as the sole C source.

3.4.3) Carbon Concentration Effects are Carbon Source Contingent

Interestingly, it does not seem that increasing glycerol abundance (11M versus 22M) correlates with an increase in USDA110 SGR. This may imply that a different nutrient, possibly phosphate, is limiting during glycerol catabolism. This observation does not hold true, however, for glucose. A significant difference ($p \leq 0.05$) was observed between the lower average SGR of the 11M glucose treatment versus the higher SGR of the 22M glucose treatment. Glycerol was demonstrated to support higher average SGRs than glucose for USDA110 populations under the tested conditions. Similarly, both concentrations of glycerol were shown to support higher average max OD_{600} values than either concentration of glucose.

Treatment	SGR (h^{-1}) Avg ± SE	Levels
11 Glycerol	0.075696 ± 0.000478	A
22 Glycerol	0.077606 ± 0.000378	A
11 Glucose	0.049559 ± 0.00026	B
22 Glucose	0.060383 ± 0.000293	C

Table3. Average specific growth rates by treatment when varying C source and concentration. Levels indicate statistical difference as determined from 1-way ANOVA and post-hoc Tukey's HSD. Levels not connected by same letter are statistically different (α=0.05).

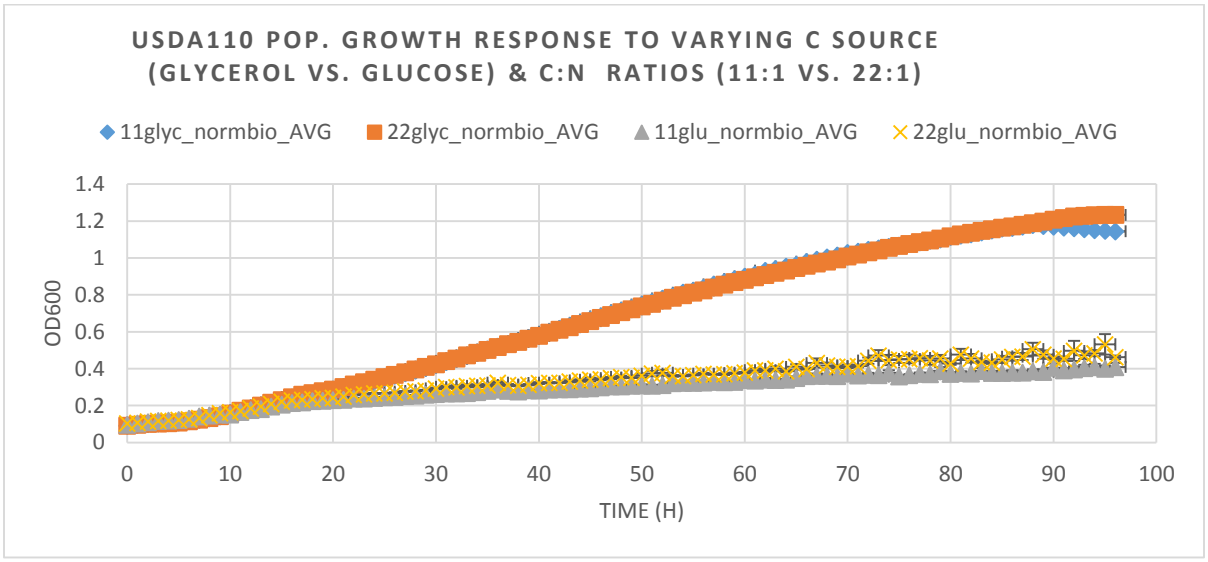

Figure 3. USDA110 population growth under two different sources of C (glycerol vs. glucose) and two different C:N ratios (11 vs.22).

3.4.4) Nitrogen Source Affects SGR and Population Yield Differently

Results of testing for growth effects caused by differences in nitrogen source (nitrate versus ammonium) showed that there was a significant difference (p=0.0081) between the average SGR of ammonium-grown (0.0655 h^{-1}) versus nitrate-grown (0.0677 h^{-1}) USDA110 populations, with nitrate producing a higher average SGR. Interestingly, the max OD_{600} also varied significantly (p=<0.0001) between ammonium-grown (0.832) and nitrate-grown (0.765) populations, however, the correlations were reversed, with ammonium producing a higher average max OD_{600}. As this experiment was conducted before empirical testing of variable phosphate concentrations suggested possible phosphate limitation, this experiment contained low phosphate (0.5 µM) levels, thus likely explaining why the max OD_{600} values are comparatively lower.

3.4.5) Phosphate Concentration as a Possible Limiting Factor

Results of testing for growth effects caused by differences in phosphate concentration showed that there was a significant difference in growth rate and max OD_{600} between all three phosphate concentrations tested (50, 5, and 0.5 µM). It appears that there is a positive relationship between increasing USDA110 population growth and increasing phosphate concentration, thereby suggesting that phosphate may be a limiting nutrient for USDA110 populations when utilizing glycerol as a sole C source and nitrate as a sole N source. Highest observed average SGR (0.075 h^{-1}) and average max OD_{600} (1.2) values correlated with the highest phosphate concentration tested (50 µM). Lowest observed average SGR (0.0675 h^{-1}) and average max OD_{600} (0.75) values correlated with the lowest phosphate concentration tested (0.5 µM). Relatedly, average SGR (0.071 h^{-1}) and average max OD_{600} (0.82) values for

the median phosphate concentration tested (5 µM) produced results that were bracketed by the values of the higher and lower phosphate treatments. As 50 µM phosphate was found to support the highest SGR and population yield values, this concentration was added to the zero-precipitate (ZY0) medium to become the finalized ZY medium. A phosphate concentration of 50 µM corresponds to a concentration commonly found in soil solutions and fertile soils (Beck and Munns, 1984; Reisenauer, 1966), and thus supports robust growth of USDA110 while remaining relevant to agricultural systems.

3.4.6) Finalized ZY Medium: Tests for Reproducibility & Format

3.4.6.1) *Reproducibility Demonstrated for Microculture Format*

The finalized ZY medium formulation – which contains glycerol as a C source, a C:N ratio of 11, nitrate as an N source at a concentration of 5.9, phosphate in a 50 µM concentration, no additional vitamins, pH = 6.6—was tested for reproducibility of USDA110 population growth over time, as understood by performing identical experiments (microtiter format, medium conditions and other experimental conditions held constant) at two different dates. No significant difference was found between independent populations of USDA110 grown in finalized ZY medium with respect to average SGR ($p=0.0812$) and average max OD_{600} ($p=0.1286$), thereby demonstrating that growing USDA110 populations in ZY medium under microtiter conditions with 200 rpm shaking, 30°C temperature, and darkness facilitates reproducible results.

3.4.6.2) *Micro VS. Midiculture Formats: Comparable SGR but not Comparable Yield*

Using finalized ZY medium, the effect of two different culture formats (96-well microtiter and 15 mL midiculture tubes) upon USDA110 population growth was examined. No significant difference was found between the average SGR of the micro-format (0.0749 h^{-1}) group versus the midi-format (0.0727 h^{-1}) group (p=0.0923). However, there was a significant difference found between the average max OD_{600} value of the micro-format (1.2) group compared to the midi-format (1.7) group (p= <0.0001), wherein a higher average max OD_{600} value in the midiculture group is possibly attributable to increased relative aeration. Work by Merrit and An (An and Friedman, 2000) posits that the adhesive "behavior" of bacterial populations (understood as adhesion to culture vessel surfaces) may differ according to vessel material (e.g. polystyrene versus borosilicate) and culture age. Within this work, it is also possible that absorbance values over time may be influenced by the nature of the culture vessel material, but no experimentation was done to investigate this further.

3.5) *In Vitro* Solid Medium Test for Citrate Utilization

A colorimetric test for citrate utilization using Simmons Citrate solid media was used to assay for potential utilization of citrate as a primary C source by USDA110. A positive result (ability to utilize citrate as a primary C source) would be indicated by a change of the medium's color from a light green to a bright blue. USDA110 was tested alongside a PG201 (a strain of *Pseudomonas aeruginosa* with a documented ability to utilize citrate as a primary C source) as a positive control. Color change for the PG201 plate was observed within 48 hours, thus confirming that the positive control worked. It was empirically confirmed (lack of color change within 7 days) that both the USDA110 single colony isolate stock, as well as

the original USDA110 stock used to produce the single colony isolate stock, were unable to utilize citrate as a primary C source (Figure 4).

Figure 4. Citrate Test for use of citrate as a primary C source. USDA110 (left panel) does not use citrate as a primary C source, resulting in a negative result with no green to blue color change. The red line on the left plate indicates that two separate stocks of USDA110 were struck on the same plate, and that both produced a negative result after 6 days of outgrowth. P. aeruginosa PG201 (right panel), was used as a positive control and was shown to induce a (blue) color change on Simmons Citrate agar thus confirming its ability to metabolize citrate as a primary carbon source.

3.6) Preliminary Testing of Copper Salts Exposure

Preliminary exposure testing of Cu salts to USDA110 populations grown to stationary phase in ZY medium was performed. Comparisons of the average SGR across all 9 treatments (control; $CuSO_4$ at 0.26, 2.6, and 26 ppm elemental Cu; $CuCl_2$ at 0.26, 2.6, and 26 ppm elemental Cu; Cu Acetate at 0.26, 2.6, and 26 ppm elemental Cu) revealed that all groups had average SGRs that varied significantly from every other group ($p \leq 0.0207$ for all pairwise comparisons). Regardless of the source of Cu salt used ($CuSO_4$, $CuCl_2$, or Cu Acetate) under conditions of low Cu exposure (0.26 ppm Cu), USDA110 populations were shown to have higher absorbance (OD_{600}) values after approximately 30 hours, thus ostensibly indicating higher population density, respective to the control (Figures 6, 7, and 8).These higher absorbance values were observed to persist past hour 30 through the duration

of the experiment to the 96th (final) hour. While the low variability between technical replicates within treatments indicates the medium and testing method's reliability and reproducibility of use, the results of the growth curves raise questions about the specifics of biotic and abiotic dynamics within a given culture well.

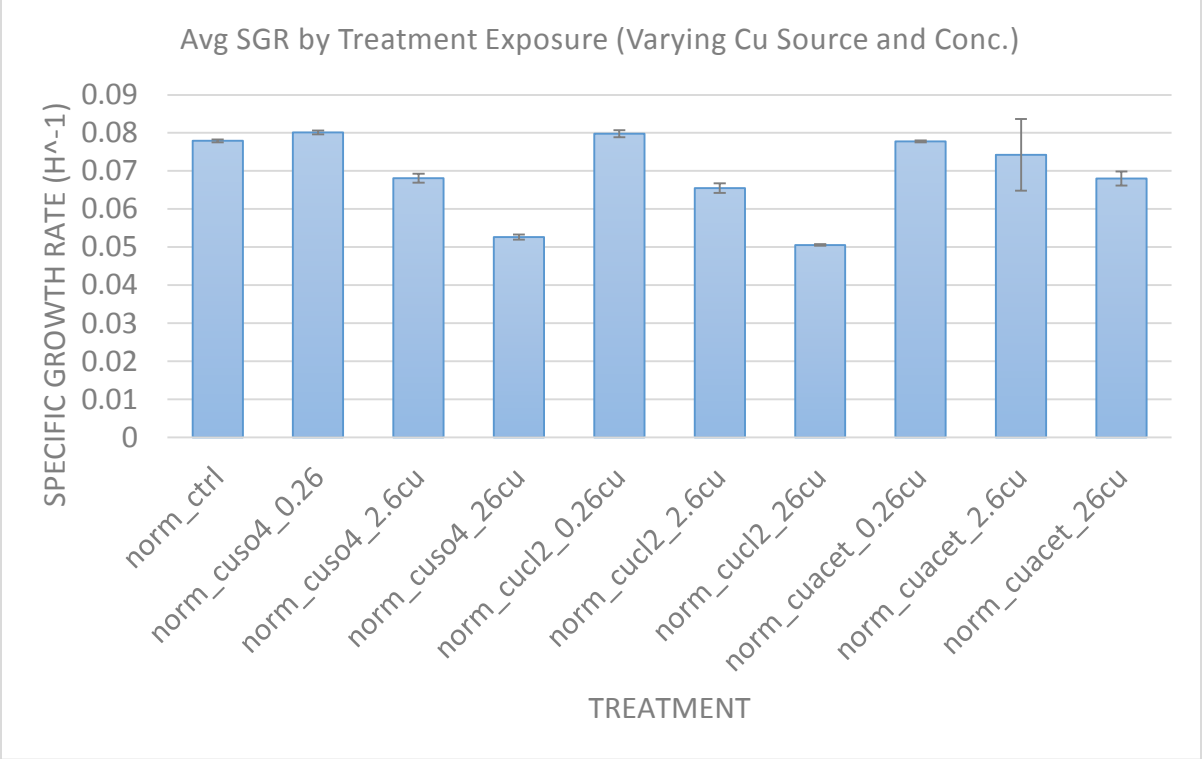

Figure 5.Average SGR values of USDA110 populations in Cu exposure treatments. In addition to a control containing no additional Cu, there were 9 exposure treatments representing three forms of Cu (CuSO4, CuCl2, and Cu Acetate) and three concentrations of Cu standardized to elemental Cu content (0.26, 2.6, and 26 ppm Cu)."Norm" refers to normalized data wherein abiotic controls have already been accounted for in the data processing.

Treatment	SGR (h^{-1}) AVG	SGR SE(±)	Max OD_{600} AVG
norm_ctrl	0.077925	0.000379	1.19
norm_cuso4_0.26cu	0.080142	0.000507	1.25
norm_cuso4_2.6cu	0.068077	0.001175	0.61
norm_cuso4_26cu	0.052639	0.000672	0.39
norm_cucl2_0.26cu	0.079798	0.000939	1.28
norm_cucl2_2.6cu	0.065514	0.001248	0.66
norm_cucl2_26cu	0.050557	0.000225	0.26
norm_cuacet_0.26cu	0.077798	0.000257	1.39
norm_cuacet_2.6cu	0.074249	0.009434	0.75
norm_cuacet_26cu	0.068024	0.001814	0.37

Table 4. Cu exposure treatments' calculated SGR averages and standard error values. In addition to a control containing no additional Cu, there were 9 exposure treatments representing three forms of Cu (CuSO4, CuCl2, and Cu Acetate) and three concentrations of Cu standardized to elemental Cu content (0.26, 2.6, and 26 ppm Cu)."Norm" refers to normalized data wherein abiotic controls have already been accounted for in the data processing.

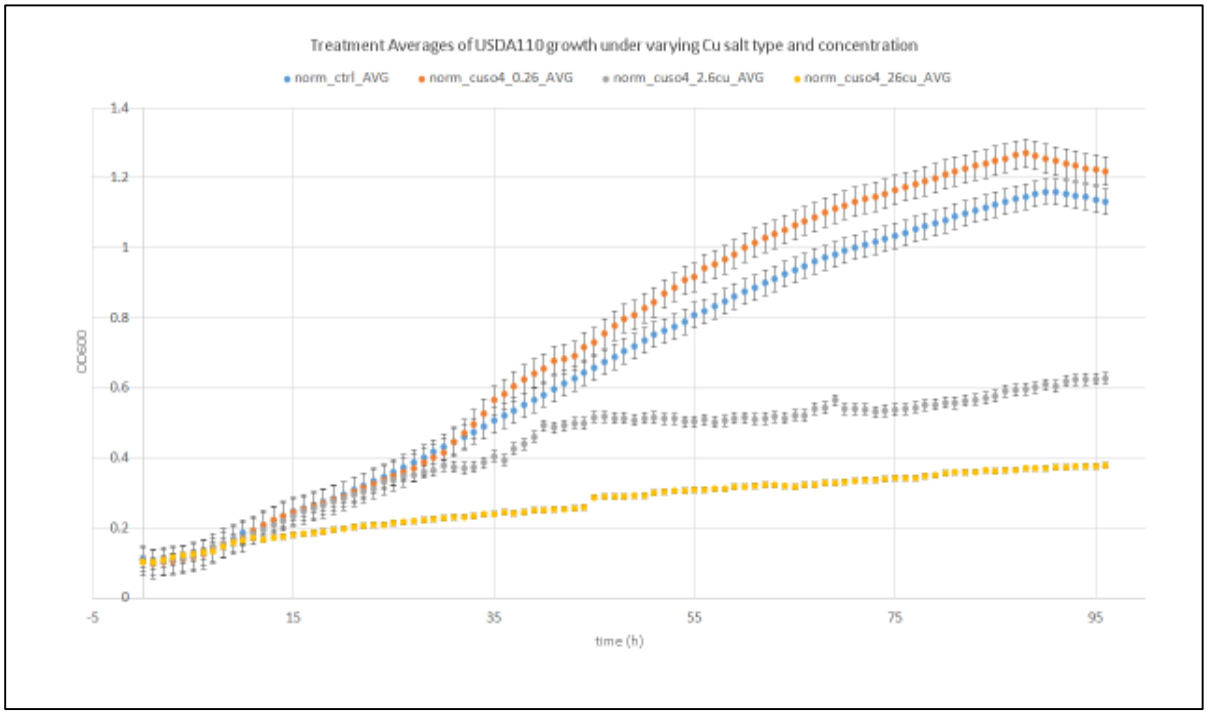

Figure 6. 95 hour USDA110 population growth when exposed to 3 different $CuSO_4$ concentrations (0.26, 2.6, 26 ppm). Light blue is control (no additional Cu) treatment.

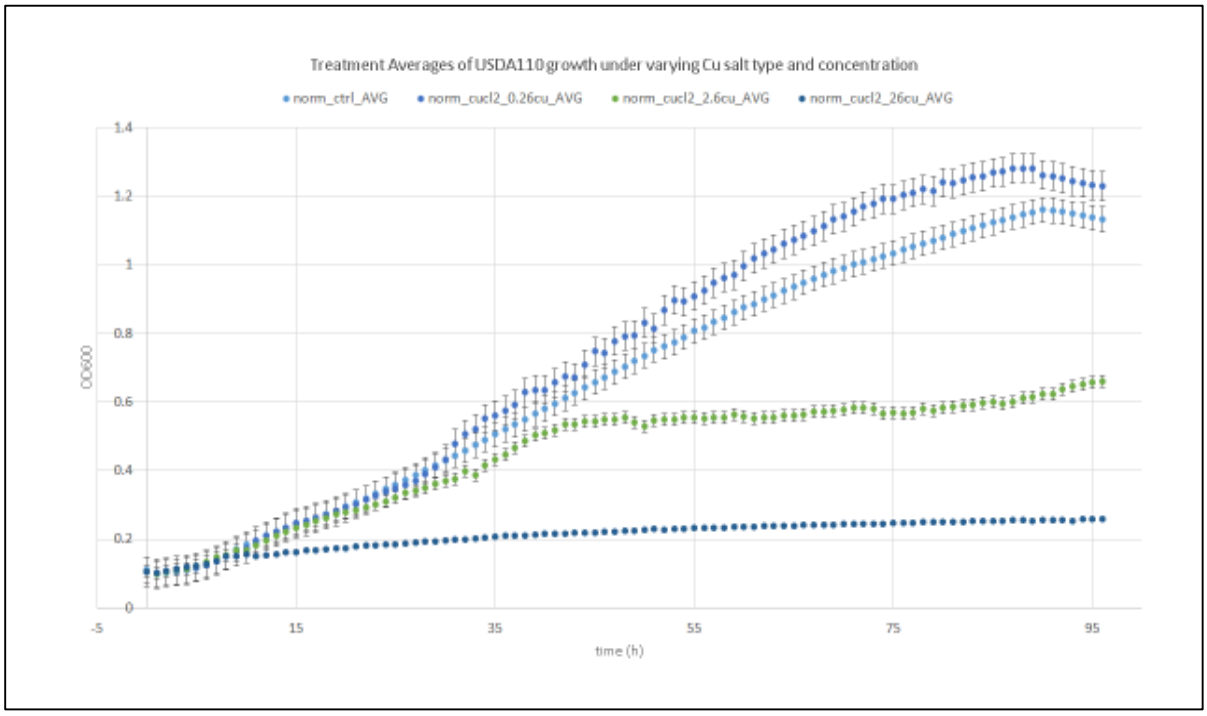

Figure 7. 95 hour USDA110 population growth when exposed to 3 different $CuCl_2$ concentrations (0.26, 2.6, 26 ppm). Light blue is control (no additional Cu) treatment.

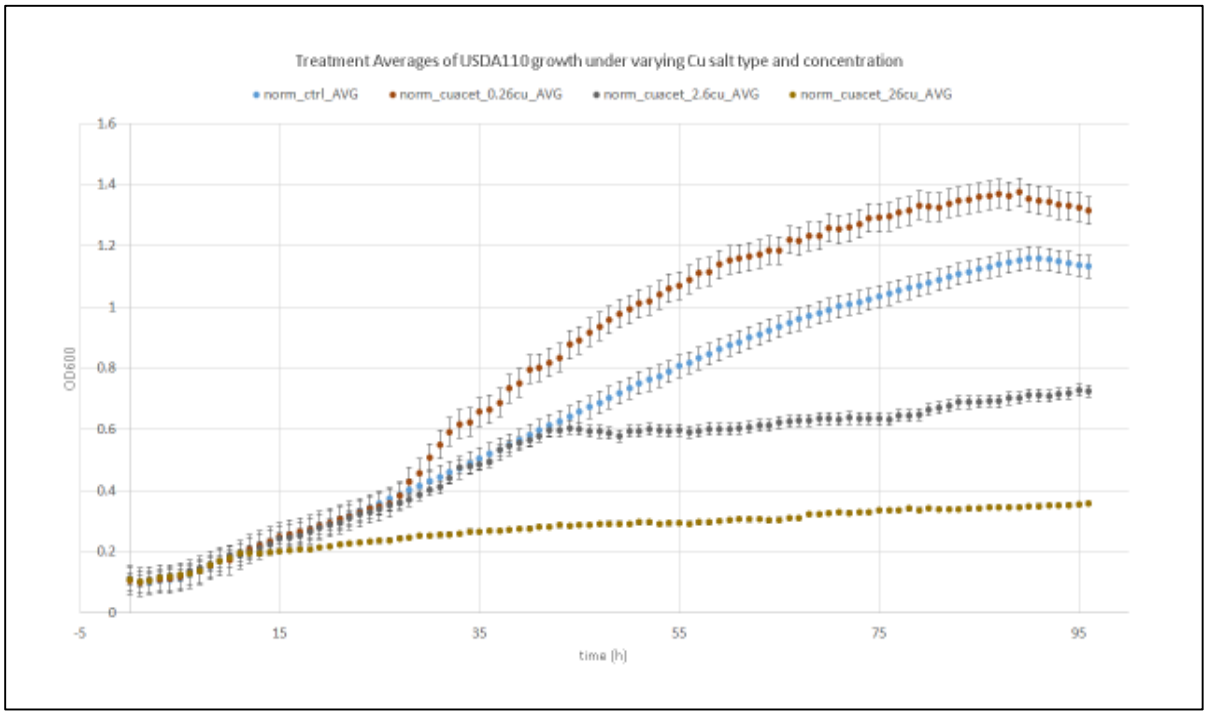

Figure 8. 95 hour USDA110 population growth when exposed to 3 different Cu Acetate concentrations (0.26,, 2.6, 26 ppm). Light blue is control (no additional Cu) treatment..

4) Discussion

Based on iterative empirical testing, the finalized ZY medium formulation demonstrates an optimization of USDA110 population growth based on the parameters tested, and a high degree of reproducibility based on within treatment and between experiment comparisons. ZY medium is able to generate SGR (range: 0.0757 – 0.0779 h^{-1}) and maximum yield (OD_{600} = ~1.2) values comparable to many other extant rhizobia culture media that have been used in the literature (Appendix A, Table C).

It is additionally worth noting the variations in effect size seen between different tested conditions, and the magnitude of the significant effects observed. When evaluating the impact of carbon sources upon growth (sections 3.4.1 and 3.4.3), the change in mean SGR

was small (under 10%) whereas the changes in maximum population yield were very large (almost a factor of two). In contrast, the vitamin effect (section 3.4.2.1) on both SGR and maximum population yield was small (under 10%). When evaluating the impact of nitrogen source, the effect size magnitude of maximum population yield was shown to be larger than that of SGR (section 3.4.4). Somewhat similarly, the effect size magnitude of maximum population yield was larger than that of SGR when evaluating the impact of phosphate concentration (section 3.4.5), though the magnitude of SGR effect size reached 10%, thus exceeding SGR magnitudes corresponding to other tested conditions. Overall, a wide range of variation in effect sizes across both metrics of SGR and maximum population yield was observed. A possible trend of maximum population yield having the larger effect size magnitudes when compared to SGR may be noted within this work; this is likely linked to physiological limitations that USDA110 possesses as a classified slow-growing rhizobium—wherein growth rate is unable to be increased beyond a certain limit, demonstrated in part by Shah and Emerich (2006), and population yield is less "constrained" and has more flexibility toward increase.

A number of factors of ZY medium's design may allow it to be used to great success within the field of metal or metal oxide-based, engineered nanoparticle (MOx ENP) toxicity testing. For instance, ZY's comparatively low IS respective to other, higher-salt, defined media may help to facilitate stable nanoparticle dispersions over time as it has been demonstrated that environments with high IS correlate with increased nanoparticle aggregation (Conway et al., 2015).

Further, unlike some other media which utilize buffers known to bind metals, the MOPS buffer included in ZY medium has very low binding affinity for metals within an

aqueous solution. MOPS lacks the hydroxyethyl or hydroxymethyl groups seemingly required for metal binding (Zhao and Chasteen, 2006). Yu et al. (1997) identifies MOPS as containing a sterically blocked tertiary amine which prevents it from complexing with metals. Additionally, Yu et al. (1997) notes that the sulfonic acid group in MOPS is such a weak nucleophile that it is unable to form a coordinate bond of observable strength. The lack of binding and interference between MOPS and copper (Mash et al., 2003) supports the idea that ZY medium may confer benefits not realized by many other media used in some toxicity testing situations involving metals and perhaps other metal-based toxicants. Toxicity results obtained through conducting growth studies in a medium with low inherent metal-binding capacity may allow researchers to worry less about underestimation of *in vitro* toxicity data when compared to "real-world" exposure scenarios. The theoretically high (bio)availability of metals within ZY medium thus could help to generate data that may be thought of as helping to inform policy and regulation based on conservative, "worst-case" impact estimates for various exposure scenarios. However, further work to empirically test for metal bioavailability and speciation would need to be performed to assess the validity of this possibility.

The empirical preliminary Cu exposure testing results included in this thesis hint at the complexity of the culture system, and the additional work needed to fully characterize interactions between USDA110 populations, the surrounding culture environment, and any introduced testing materials. As ZY medium contains Cu in amounts known to be biologically beneficial to the growth of Bradyrhizobia, and as MINEQL+ modeling does not predict Cu-based precipitates to form, it is unlikely that Cu levels in the medium are insufficient and that the USDA110 populations are experiencing a stimulatory effect from the

presence of the added 0.26 ppm Cu. Therefore, possible, alternate explanations that acknowledge both biotic and abiotic factors should be considered.

Various cellular physiological and/or morphological changes may be occurring within the USDA110 population over time when under prolonged Cu exposure. These changes in rhizobia may include increased production of extracellular polymeric substances (Nocelli et al., 2016) development of a "permeability barrier" composed of cell surface proteins (Tindwa et al., 2010), or entrance into a semi-stasis state termed Viable But Non-Culturable (VBNC) (Alexander et al., 1999). It is currently unknown how any of these possible phenomenon may, by acting alone or in concert, alter the optical characteristics of USDA110 populations, thus influencing absorbance readings over time and decoupling the relationship between optical density and population density. Further work is needed in order to fully explore and describe the culture dynamics of USDA110 populations over time, especially under conditions of metal exposure.

5) Conclusions

The use of ZY medium in a microtiter culture format to undertake USDA110 growth studies offers advantages that may benefit ecological toxicity testing and risk assessment given certain considerations. Empirical evidence from USDA110 growth studies demonstrates that populations of planktonic USDA110 are able to grow well in ZY medium—achieving a high degree of within and between study reproducibility, and sufficiently high absorbance values allowing for comparable analysis. Reliable and

reproducible growth in microtiter format enables studies to be done automated testing conditions that are likely to prove both time and money-saving.

As ZY medium's formulation was based on considerations for environmentally-comparable soil nutrient levels and rhizobial biological requirements, ZY medium facilitates testable, experimental conditions that are better representative of environmentally-relevant, nutrient-limited scenarios that still support USDA110 population growth. A comparison between reported values of rhizobial biological nutrient requirements and calculated nutrient solubilities (Table 1) was performed originally, and provided the base foundation for the medium's design. Subsequent chemical speciation modeling calculations (MINEQL+) elucidated the reagents responsible for precipitate formation within the medium, thus enabling a "fine-tuning" approach in regards to reducing metal and phosphate-based precipitate formation. In this manner, a zero-predicted precipitate medium (ZY0) formulation was pinpointed.

Empirical testing allowed for investigation of the response of USDA110 populations' specific growth rate and maximum yield to the sole varying of phosphate concentration. This investigation revealed that while the zero-predicted precipitate medium (ZY0) was able to support adequate USDA110 population growth, a strong correlation existed between increasing phosphate concentration and both increasing SGR and maximum yield. Precipitation of solids in the medium was not observable when phosphate concentration was increased to 50 µM, thereby signifying that actual precipitates either do not occur to the degree they are predicted, or that such levels are not visualizable with the naked eye, thus again calling into question the formulation of other "zero precipitate" growth media. The final ZY medium formulation contains phosphate at a level comparable to those of natural

soils; this concentration represents a compromise between low phosphate-based precipitate formation and a high degree of support for USDA110's nutrient requirements.

Empirical testing provided confirmation for ZY's ability to support robust planktonic USDA110 population growth at measurements of up to 1.2 OD_{600} in microculture and 1.7 OD_{600} in midiculture. As ZY medium is able to support robust USDA110 population growth while mitigating some problems inherent to other rhizobial growth media (undefined composition; inclusion of problematic reagents), ZY medium may be used as a possible alternative medium with which to conduct *in vitro* USDA110 growth and testing studies. These results improve upon existing culture and growth study methodology, and provide a basis for considering the many factors inherent to best approaches in growth medium design toward predictive toxicity testing.

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APPENDIX A: ADDITIONAL TABLES & FIGURES

Table A. MINEQL+ ZY medium reagent input (ionic concentrations) and precipitate output.

To use MINEQL+ software for predictive equilibrium speciation modeling, one must divide all reagents into their correspondent ionic constituents, and then calculate the sum total M of all ionic species present. These combined M values are then entered into MINEQL+ as input data alongside experimental conditions such as temperature, pH, and carbonate system chemistry. The conclusion of modeling provides output data in the form of various classes of predicted media fractions: components, complexes, fixed entities, precipitated solids, and dissolved solids. Output data for ZY showed $MnHPO_4$ as the sole predicted precipitated solid; regular visual checks of the medium (same batch over time; across batches over time) showed no visualizable precipitation of solids in the medium.

ZY Input Data:

Ions	total [M]
Na^{+1}	9.41E-03
PO_4^{-3}	5.00E-05
Ca^{+2}	0.000021
Cl^{-1}	0.003757
MoO_4^{-2}	2.66E-05
Mg^{+2}	0.0016
SO_4^{-2}	0.004929
Fe^{2+}	0.00002
K^{+1}	0.006835
NO_3^{-1}	0.005935
Zn^{+2}	4E-07
Mn^{+2}	0.000004
Cu^{+2}	1.57E-06
$B_4O_7^{-2}$	4.46E-07
Co^{+2}	8.89E-07
citrate ($C_6H_5O_7$)	0.001938
$EDTA^{-4}$	7.44E-06
MOPS ($C_7H_{15}NO_4S$)	0.04
glycerol ($C_3H_8O_3$)	0.021718
**CO_3^{-2} b/c open system (log $_PCO_2$)	-3.5
pH	6.6

ZY Output Data:

Predicted Precipitated Solids

Precipitate	Conc [M]
$MnHPO_4$	3.73E-06

Table B. ZY Medium Reagent Concentrations, Molecular Weights, and Calculated Ionic Strengths

Reagent	Reagent M	Molec Weight	Ionic Strength (M)
Na2HPO4	5.00E-05	141.9588	0.0006
CaCl2*2H2O	0.000021	147.0146	0.000126
NaCl	0.0034255	58.4428	0.006851002
Na2MoO4*2H2O	2.9396E-05	241.9677	0.000176374
MgSO4	0.002	120.366	0.016
KNO3	0.00593453	101.1032	0.011869061
ZnSO4*7H2O	0.0000004	287.5496	0.0000032
MnSO4*H2O	0.000004	169.0159	0.000032
CuSO4	1.5726E-06	159.609	1.25808E-05
Na2B4O7*10H2O	4.7435E-07	381.3721	2.84612E-06
CoSO4*7H2O	8.8935E-07	281.0928	7.11483E-06
diSodium EDTA dihydrate	7.4359E-06	372.24	0.000133847
FeSO4*7H2O	0.00002	278.02	0.00016
sodium citrate dihydrate (Na3C6H5O7*2H2O)	0.00193753	294.1	0.023250407
glycerol	0.02171788	92.09	non-ionic kosmotrope
MOPS	0.04	209.2633	0.007
			Total = 0.033112216

The equation used for calculating Ionic Strength (IS) is as follows, wherein c_i is the molar concentration of ion "i," z_i corresponds to the charge of ion "i," the sum is taken over all the ions present in the solution, and then halved to account for inclusion of both anions and cations.

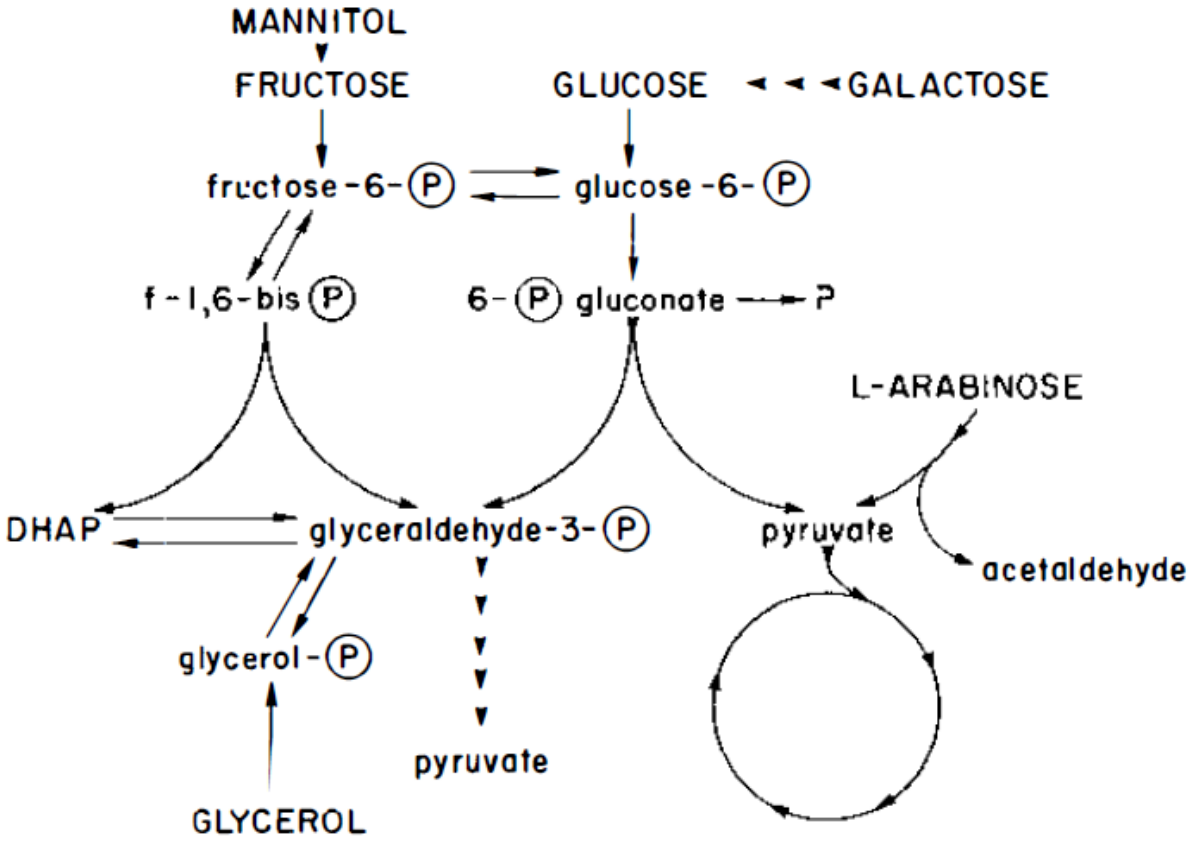
$$I = \frac{1}{2}\sum_{i=1}^{n} c_i {z_i}^2$$

Table C. Comparison of calculated SGRs derived from previous USDA110 growth experiments found in the literature against USDA110 SGR's observed with ZY Medium.

The experimental conditions in the listed papers varied greatly, and the media used represent both undefined (Yoshida et al., 2013) and defined (Green and Emerich, 1997) types. The SGRs from the other papers listed were not reported as such, and were created for this work by myself via extrapolated calculation. Thus, they are best-effort approximations representing only one dataset, and therefore do not have concordant standard deviation or standard error values.

SOURCE PAPER & RELEVANT FIGURE	**SGR (H^{-1})**
Masuda et al. 2010. Fig 1B	0.045
Plessner et al. 1993. Fig 1	0.0836
Plessner et al. 1993. Fig 1	0.0885
Green and Emerich. 1997. Fig 4	0.0925
Green and Emerich. 1997. Fig 4	0.0549
Green and Emerich. 1997. Fig 4	0.0957
Yoshida et al. 2013. Fig 3	0.0363
Yoshida et al. 2013. Fig 3	0.0508
Yoshida et al. 2013. Fig 3	0.0520
Hohle and Thomas 2012. Fig 7	0.0943
ZY medium (control treatment)	0.0757 – 0.0779

Figure A. Metabolic pathways for C catabolism in slow-growing rhizobia (from Stowers, 1985). Permission to republish kindly enabled by MD Stowers, the Annual Review of Microbiology, Annual Reviews, and Copyright Clearance Center, Inc.



APPENDIX B: CUSTOM PYTHON SCRIPT (SPEKIT V0.2.2) SOURCE CODE

```
#!/usr/bin/env python

#Ver. 0.2.2

from itertools import islice
from pandas import *
import numpy as np
import matplotlib
matplotlib.use('gtkAgg')
import matplotlib.pyplot as plt
from pylab import *
from math import log
import os
import multiprocessing
from scipy import stats
import time
from multiprocessing import Process, Pipe
import sys
import gobject
from matplotlib.ticker import ScalarFormatter, FormatStrFormatter
from decimal import *
import os, errno
import argparse
```

```
import ntpath
import pipes
import pygtk
import gtk
import copy


class spekit(object):
    def __init__(self,o,i,winrange,quick,auto,noplot,nosave,skiptocol,hardcap):
        self.examine = False
        self.editing = False
        self.plotopen = False
        self.hardcap = hardcap
        self.wrange = winrange
        self.edit_defaults = {"1":['Cap','Unlocked',0,'No Cap'],
                              "2":['Anchor','Unlocked',False,'No Anchor'],
                              "3":['Resort','Unlocked',False,'Slope of Ln'],
                              "4":['Window','Unlocked',winrange,winrange[0]],
                              "5":['Manual','Unlocked',False,'False']}
        self.set_edits = copy.deepcopy(self.edit_defaults)
        self.bypasslevel = [quick,auto,noplot,nosave]
        self.data_input = i
        self.date_stamp = time.strftime("%d_%m_%Y")
        if skiptocol != 0:
            self.colnum = skiptocol - 1
            self.i = skiptocol - 1
```

```
        else:
            self.colnum = 0
            self.i = 0
        self.xx = DataFrame.from_csv(self.data_input, sep =',')
        self.plot_pipe, self.plotter_pipe = Pipe()
        self.send = self.plot_pipe.send
        self.final_dict = {}
        self.wrange = winrange
        self.cap = self.hardcap
        if o == None:
            self.out_dir =
os.path.normpath(os.path.join(os.path.abspath(self.data_input),'..','spekit_{0}'.format(os.path.
splitext(ntpath.basename(i))[0])))
        else:
            self.out_dir =
os.path.normpath(os.path.join(o,'spekit_{0}'.format(os.path.splitext(ntpath.basename(i))[0]))
)
        if os.path.exists(self.out_dir):
            i=0
            while os.path.exists("{0}_{1}".format(self.out_dir,i))==True:
                i+=1
            if not os.path.exists("{0}_{1}".format(self.out_dir,i)):
                self.out_dir = "{0}_{1}".format(self.out_dir,i)
                self.mkdir_p(self.out_dir)
        else:
            self.mkdir_p(self.out_dir)
        self.out_file = os.path.join(self.out_dir,"Full_output.csv")
```

```
        x = open(self.out_file,'wb')
        x.write("Run Name,Specific Growth Rate,Lag Time, Window Size, StartX, StartY, StopX, StopY, Slope Equation (non-log), Slope Equation(Ln), Choice Selected\n")
        x.close()
        x = open(os.path.join(self.out_dir,"COMMANDS.txt"),'w')
        x.write("python ")
        for y in sys.argv:
            x.write("{0} ".format(y))

        x.close()
        self.reset()
        self.start()

    def start(self):
        while self.i < len(self.xx.columns):
            self.cap = self.set_edits['1'][2]
            self.plotopen = False
            run_name = self.xx.columns[self.colnum]
            self.save_file = os.path.join(self.out_dir,run_name)
            self.save_file = os.path.normpath(self.save_file)
            self.temp_choice = None
            self.winnum = 0
            self.logtime = False
            self.winList = []
            self.windex = {}
```

```
        self.unlogwindex = {}

        self.windowDict = {}

        self.hiddenwindex = {}

        self.intDict = {}

        self.winrange = self.set_edits['4'][2][0]

        self.winmax = self.set_edits['4'][2][1]

        print("\nYou are currently analyzing column #{0} of {1} columns.
({2})".format(self.colnum+1,len(self.xx.columns),self.xx.columns[self.colnum]))

        txt = self.set_edits

        print("\nSorting by '{0}' | Anchor: '{1}' | Cap: '{2}' | MinWin: '{3}' | Manual
'{4}'".format(txt['3'][3],
                                                                    txt['2'][3],

                                                                    txt['1'][3],

                                                                    txt['4'][3],

                                                                    txt['5'][3]))

        if self.set_edits['5'][2]:

            self.set_edits['5'][2] =
list(self.find_anchor_head(self.set_edits['5'][2][0],self.set_edits['5'][2][1]))

        if self.set_edits['2'][2]:

            self.set_edits['2'][2] = self.find_anchor_head(self.set_edits['2'][2])

        while self.winrange <= self.winmax:

            self.analyze_window(self.windowed(n=self.winrange))

            self.winrange+=1

        choice_fragment = open("{0}_Choices.csv".format(self.save_file), 'wb')

        choice_fragment.write("{0},{1},{2},{3},{4}\n".format("Choice","R2","Slope","Win
Start","Win End"))

        cx = 1
```

```
for wi in self.choiceDF.index[:20]:
    if cx <= 4:
        self.winList.append(self.windowDict[wi])
        cx += 1
    choice_fragment.write("{0},{1},{2},{3},{4}\n".format(wi,
                                                         self.choiceDF["R2"][wi],
                                                         self.choiceDF["Slope"][wi],
                                                         min(self.windowDict[wi]),
                                                         max(self.windowDict[wi])))
choice_fragment.close()
print ("Plotting..")
if self.set_edits['5'][2] != False:
    print self.choiceDF.ix[self.manual_selection]
    self.temp_choice = self.manual_selection
    self.logtime= True
    self.plot_type = 'single'
    self.plot_prep()

    self.singlechoice_final()
elif len(self.choiceDF.index) < 4 and self.editing == True:
    print ("\nResult list too small to graph. Please examine individually\n")
    self.logtime = True
    self.singlechoice()
else:
    self.plot_quad()
```

```
            self.choose()
        self.save_vars()


def save_vars(self):
    if self.i < len(self.xx.columns):
        self.colnum += 1
        with open(self.out_file,'a') as outfile:
            for key in self.final_dict:
                outfile.write("{0},{1},{2},{3},{4},{5},{6},{7},{8},{9},{10}\n".format(key,
                                                        self.final_dict[key][0],
                                                       self.final_dict[key][1],
                                                       self.final_dict[key][2],
                                                       self.final_dict[key][3],
                                                       self.final_dict[key][4],
                                                       self.final_dict[key][5],
                                                       self.final_dict[key][6],
                                                       self.final_dict[key][7],
                                                            self.final_dict[key][8],
                                                                self.final_dict[key][9]))
        self.final_dict = {}
        if self.bypasslevel[3] == False:
            print("Saving..")
            time.sleep(3)
            print("Saved!")
        print("\n################################\n")
```

```
        self.i += 1
        for a in self.set_edits.keys():
            if self.set_edits[a][1] == 'Unlocked':
                self.set_edits[a] = copy.deepcopy(self.edit_defaults[a])
        time.sleep(1)
        self.start()

def find_anchor_head(self,anchor,head=False):
    win_min_dict = {}
    win_max_dict = {}
    for x in self.xx.index:
        win_min_dict.setdefault(abs(x-anchor),x)
        if head:
            win_max_dict.setdefault(abs(x-head),x)
    if head:
        return(win_min_dict[min(win_min_dict)],win_max_dict[min(win_max_dict)])
    else:
        return(win_min_dict[min(win_min_dict)])

def plot_quad(self):
    self.plot_type = 'quad'
    self.logtime = True
    self.plot_prep()
```

```
def reset(self):
    print "resetting"
    if self.editing == False:
        print "inside"
        self.cap = self.hardcap
        self.anchor_num = False
        self.resort_num = False
        self.manual_num = False
        self.set_window_num = False
        self.set_window_txt = self.wrange[0]
        self.manual_txt = "False"
        self.cap_txt = "No cap"
        self.anchor_txt = "No Anchor"
        self.resort_txt = "Slope of Ln"
        print "done setting"


def choose(self):
    if len(self.choiceDF.index) > 8:
        print(self.choiceDF[0:8])
    else:
        print(self.choiceDF)
    if self.bypasslevel[1] == True:
        self.temp_choice = self.winList[0][0]
        self.singlechoice_final()
    else:
```

```
            self.choice = raw_input("Which set would you like to keep?\nTo view more sets or
enter Editing mode enter '-'.\nspekit> ")
            if self.choice == '-':
                self.examine = True
                self.edit_mode()
            else:
                try:
                    self.examine = False
                    self.temp_choice = int(self.choice)
                    self.close_graph()


                    self.plot_type = 'single'
                    self.plot_prep()


                    self.singlechoice_final()
                except:
                    print("You have made an invalid selection.")
                    self.choose()



    def plot_prep(self,finished=False):
        if self.bypasslevel[3] != True and self.plotopen==False:
            self.data = []
            self.plotter = ProcessPlotter()
            self.plot_process = Process(target = self.plotter,args = (self.plotter_pipe,
```

```
                                                self.plot_type,
                                                self.lnDF,
                                                self.winList,
                                                self.colnum,
                                                self.windowDict,
                                                self.temp_choice,
                                                self.choiceDF,
                                                self.logtime,
                                                self.xx,
                                                self.intDict,
                                                self.save_file,
                                                self.bypasslevel,
                                                self.examine))
            self.plot_process.daemon = True
            self.plotopen = True
            self.plot_process.start()


def edit_mode(self):
    self.editing = True
    tlist = []
    for x in self.set_edits.keys():
        if x == '4':
            if self.set_edits[x][1] == 'Locked':
                tlist.append("{0}*".format(self.set_edits[x][2]))
            else:
```

```
                tlist.append(self.set_edits[x][2])
            continue
        if self.set_edits[x][1] == 'Locked':
            tlist.append("{0}*".format(self.set_edits[x][3]))
        else:
            tlist.append(self.set_edits[x][3])
    print("\nCurrent Edits:")
    print("Win: {0} | Anchor: {1} | Cap: {2} | Sort: {3} | Manual: {4}".format(tlist[4],
                                                                              tlist[2],
                                                                              tlist[0],
                                                                              tlist[1],
                                                                              tlist[3]))
    self.e_mode_choice = raw_input("\nPlease select a command from the following list:\
\n1. Single: View and select a single window from an expanded list.\
\n2. Cap:    Exclude windows with time points higher than a certain value.\
\n3. Anchor: Only consider windows with time points starting at a certain value.\
\n4. Resort: Re-sort the windows based on a different criteria [R2 | Slope].\
\n5. Window: Set the Max/Min window size.\
\n6. Manual: Manually select window.\
\n7. Lock/Unlock: Lock and unlock edits.\
\n8. Clear: Clear all or some of your current edits.\
\n9. Start: Re-run with the current edits.\
\nspekit> ")
    try:
        self.e_mode_choice = int(self.e_mode_choice)
```

```
except:
    print("\nERROR: Invalid selection. Please use a single, whole number.\n")
    self.edit_mode()
if self.e_mode_choice == 1:
    self.singlechoice()
    self.save_vars()
elif self.e_mode_choice == 2:
    self.cap_func()
    self.edit_mode()
elif self.e_mode_choice == 3 :
    self.anchor()
    self.edit_mode()
elif self.e_mode_choice == 4 :
    self.resort()
    self.edit_mode()
elif self.e_mode_choice == 5:
    self.set_window()
    self.edit_mode()
elif self.e_mode_choice == 6:
    self.manual()
    self.edit_mode()
elif self.e_mode_choice == 7:
    self.lock_edits()
    self.edit_mode()
elif self.e_mode_choice == 8:
```

```
            self.clear_edits()
            self.edit_mode()
        elif self.e_mode_choice == 9:
            self.close_graph()
            self.start()
        else:
            print("\nERROR: Invalid selection, Please use a selection provided.\n")
            self.edit_mode()


    def clear_edits(self):
        print("\nSelect the number of the edits to clear. Separate multiple edits with commas, no spaces.")
        clear_input = raw_input("1. Cap\n2. Anchor\n3. Resort\n4. Window\n5. Manual\n6. All\nspekit> ")
        clear_list = clear_input.split(",")
        for a in clear_list:
            if a == '6':
                for var in self.set_edits.keys():
                    if self.set_edits[var][2] != self.edit_defaults[var][2] or self.set_edits[var][3] != self.edit_defaults[var][3]:
                        self.set_edits[var] = copy.deepcopy(self.edit_defaults[var])
                print("Cleared all.")
                time.sleep(1)
                continue
            if a == '5':
                    self.set_edits['1'] = copy.deepcopy(self.edit_defaults['1'])
```

```
            self.set_edits['2'] = copy.deepcopy(self.edit_defaults['2'])
        if self.set_edits[a][2] != self.edit_defaults[a][2]:
            print self.set_edits[a]
            print self.edit_defaults[a]
            self.set_edits[a] = copy.deepcopy(self.edit_defaults[a])
            print("Cleared {0}.".format(self.set_edits[a][0]))
            time.sleep(0.5)


def cap_func(self):
    try:
        x = float(raw_input("\nValue to cap search at:  "))
    except:
        print("\nERROR:Invalid entry, please use a number, Decimals are ok.\n")
        self.edit_mode()
    self.cap = x
    self.set_edits[str(self.e_mode_choice-1)][2] = self.cap
    self.set_edits[str(self.e_mode_choice-1)][3] = self.cap


def anchor(self):
    try:
        x = float(raw_input("\nValue to anchor search at:  "))
    except:
        print("\nERROR:Invalid entry, please use a number, Decimals are ok.\n")
        self.edit_mode()
    self.set_edits[str(self.e_mode_choice-1)][2] = x
```

```
    self.set_edits[str(self.e_mode_choice-1)][3] = x


def manual(self):
    tman = []
    try:
        tman.append(float(raw_input("\nSet MINimum time point: ")))
        tman.append(float(raw_input("Set MAXimum time point: ")))
    except:
        print("\nERROR:Invalid entry, please use a number, Decimals are ok.\n")
        self.edit_mode()
    x = tman[1]-tman[0]
    if x <= 2:
        print("\nYour Max/Min are too close together!\n")
        self.edit_mode()
    else:
        self.set_edits[str(self.e_mode_choice-1)][2] = tman
        self.set_edits[str(self.e_mode_choice-1)][3] = 'True'
        self.set_edits['1'][3] = '{0}*'.format(tman[1])
        self.set_edits['2'][3] = '{0}'.format(tman[0])


def resort(self):
    print("\nSelect value to sort by:")
    print("1. R2")
    print("2. Slope of Ln")
    x = raw_input("spekit> ")
```

```
    if not x in ['1','2']:
        print("\nERROR: Please use the numbered selection [1 or 2]!\n")
        self.edit_mode()
    else:
        self.set_edits['3'][2] = x
        if x == '1':
            self.set_edits['3'][3] = 'R2'
        else:
            self.set_edits['3'][3] = 'Slope of Ln'


def set_window(self):
    x = []
    try:
        x.append(int(raw_input("\nSet MINimum window size: ")))
        x.append(int(raw_input("Set MAXimum window size: ")))
    except:
        print("\nERROR:Invalid entry, please use whole numbers\n")
        self.edit_mode()
    dist = x[1]-x[0]
    if dist < 0:
        print("\nYour Window Min is greater than your Window Max.")
        self.edit_mode()
    else:
        self.set_edits['4'][2] = x
        self.set_edits['4'][3] = x[0]
```

```
    def lock_edits(self):
        print("\nSelect the number of the edits to lock/unlock. Separate multiple edits with commas, no spaces.")
        lock_input = raw_input("1. Cap  [{0}]\n\
2. Anchor  [{1}]\n\
3. Resort  [{2}]\n\
4. Window  [{3}]\n\
5. Lock All\n\
6. Unlock All\n\
spekit> ".format(self.set_edits['1'][1],
                 self.set_edits['2'][1],
                 self.set_edits['3'][1],
                 self.set_edits['4'][1]))
        lock_list = lock_input.split(",")
        for a in lock_list:
            if a == '-':
                self.edit_mode()
            if a == '6':
                for var in self.set_edits.keys():
                    if self.set_edits[var][1] != 'Unlocked' and var != '5':
                        self.set_edits[var][1] = 'Unlocked'
                print("Unlocked all.")
                time.sleep(1)
                continue
```

```
        if a == '5':
            for var in self.set_edits.keys():
                if self.set_edits[var][1] != 'Locked' and var != '5':
                    self.set_edits[var][1] = 'Locked'
            print("Locked all.")
            time.sleep(1)
            continue
        if self.set_edits[a][1] == 'Unlocked':
            self.set_edits[a][1] = 'Locked'
            print("Locked {0}.".format(self.set_edits[a][0]))
            time.sleep(0.5)
        else:
            self.set_edits[a][1] = 'Unlocked'
            print("Unlocked {0}.".format(self.set_edits[a][0]))
            time.sleep(0.5)


def singlechoice(self):
    if len(self.choiceDF.index) > 4:
        self.plot_quad()
    if len(self.choiceDF.index) > 8:
        print(self.choiceDF[0:15])
    else:
        print(self.choiceDF)
    try:
```

```
            df_draw = raw_input("Enter a choice from the list to see it graphed. To enter Editing mode, type '-':\nspekit> ")
            if df_draw == '-':
                self.edit_mode()
            self.close_graph()

            self.temp_choice = int(df_draw)
            self.plot_type = 'single'
            self.plot_prep()

            self.singlechoice_final()
        except:
            print("You have made an invalid selection")
            time.sleep(1)
            self.singlechoice()

    def singlechoice_final(self):
        if self.bypasslevel[1] == True or self.bypasslevel[0] == True and self.examine == False:
            df_choose = 'y'
        else:
            df_choose = raw_input("To accept/reject the selection, type 'yes/no'. To enter Editing mode, type '-':\nspekit>  ")
            self.close_graph()
        if df_choose in ['y','Y','yes','Yes']:
            final_data = [self.windex[self.temp_choice][1],
                          self.unlogwindex[self.temp_choice][2],
```

```
                    len(self.windowDict[self.temp_choice]),
                    min(self.windowDict[self.temp_choice]),

self.xx.loc[min(self.windowDict[self.temp_choice])][self.xx.columns[self.colnum]],
                    max(self.windowDict[self.temp_choice]),

self.xx.loc[max(self.windowDict[self.temp_choice])][self.xx.columns[self.colnum]],

"y={0}x+{1}".format(self.unlogwindex[self.temp_choice][0],self.unlogwindex[self.temp_choice][1]),

"y={0}x+{1}".format(self.hiddenwindex[self.temp_choice][0],self.hiddenwindex[self.temp_choice][1]),
                    self.temp_choice]
            self.logtime = False
            self.final_dict.setdefault(self.xx.columns[self.colnum],final_data)
            self.plot_type = 'final'
            self.plot_prep()
        elif df_choose in ['n','N','no','No']:
            print("resetting selection")
            self.close_graph()
            self.singlechoice()
        elif df_choose == '-':
            self.close_graph()
            if len(self.choiceDF.index) > 3:
                self.plot_quad()
            self.edit_mode()
```

```
        else:
            print("Please enter 'y' or 'n'")
            time.sleep(0.2)
            self.singlechoice_final()

    def windowed(self,n=8):
        "Returns a sliding window (of width n) over data from the iterable"
        "   s -> (s0,s1,...s[n-1]), (s1,s2,...,sn), ...                   "
        #have it slice index, then pass the generator into analyze_window
        it = iter(self.xx.index)
        self.result = tuple(islice(it, n))
        if len(self.result) == n:
            yield self.result
        for elem in it:
            self.result = self.result[1:] + (elem,)
            yield self.result

    def save_window(self,window):
            self.windowDict.setdefault(self.winnum,window)
            #yax = self.xx[self.xx.columns[self.colnum]][min(window)-1:max(window)]
            yax = self.xx.ix[window,self.colnum]
            xax = yax.index
            slope, intercept, r_value, p_value, std_err = stats.linregress(xax, np.log(yax))
            self.windex.setdefault(self.winnum,[r_value**2,
slope,len(window),window[0],window[-1]])
```

```
            self.hiddenwindex.setdefault(self.winnum,[slope, intercept])
            x = (intercept*-1)/slope
            self.intDict.setdefault(self.winnum,[x])
            self.intDict.setdefault(self.winnum,).append([slope,r_value**2,intercept])
            slope, intercept, r_value, p_value, std_err = stats.linregress(xax, yax)
            x = (intercept*-1)/slope
            self.unlogwindex.setdefault(self.winnum,[slope,intercept,x,window])
            self.intDict.setdefault(self.winnum,).append(x)
            self.intDict.setdefault(self.winnum,).append([slope,r_value**2,intercept])
            self.winnum +=1


    def analyze_window(self,generator):
        list_of_windows = list(generator)
        for window in list_of_windows:
                if min(window) == self.set_edits['2'][2] or self.set_edits['2'][2] == False:
                    if self.cap == 0 or max(window) <= float(self.cap):
                        if self.set_edits['5'][2]!= False and min(window)== self.set_edits['5'][2][0]
and max(window)== self.set_edits['5'][2][1]:
                            self.manual_selection = self.winnum
                        self.save_window(window)
        if len(self.windex)==0:
            print "\n\nYour cap was set too low! either lower the window size or increase your
cap.\n\n"
            self.cap = 0
            self.edit_mode()
        self.choiceDF = DataFrame.from_dict(self.windex,orient = 'index')
```

```
        self.choiceDF.columns = ["R2","Slope","Win Size","Win Start","Win End"]
        if self.set_edits['3'][2] == False or self.set_edits['3'][2] == '2':
            self.choiceDF.sort(['Slope','R2'], ascending = [0,0],inplace=True)
        else:
            self.choiceDF.sort(['R2','Slope'], ascending = [0,0],inplace=True)
        self.lnDF = self.xx.apply(np.log,0)


    def mkdir_p(self,path):
        try:
            os.makedirs(path)
        except OSError as exc: # Python >2.5
            if exc.errno == errno.EEXIST:
                pass
            else: raise
        return(path)


    def close_graph(self):
        if self.plotopen == True:
            self.send(None)
            self.plotopen = False
            time.sleep(0.5)


################################################################################
################################################################################
########################
```

```
class ProcessPlotter(object):

    def __init__(self):
        self.x = []
        self.y = []

    def terminate(self):
        plt.close('all')

    def poll_draw(self):
        #print("CCCC")
        def call_back():
            #print("BBB")
            while 1:
                if not self.pipe.poll():
                    break

                command = self.pipe.recv()
                #print("AAA")

                if command is None:
                    self.terminate()
                    return False

                else:
```

```
                #print("AAAAAC")
                pass


            return True


        return call_back


    def singleplot(self):
        if self.logtime == False:
            self.window = 3
        else:
            self.window = 1
        getcontext().prec = 3
        fig = plt.figure(figsize=(8.0,8.0))
        self.axis_assign()
        self.ax = fig.add_subplot(111)
        self.create_plot(self.ax)
        xs,ys = self.fit_fn(self.yaxis.index, self.yaxis, self.temp_choice)
        self.ax.plot(xs,ys,'k-')
        self.keypos = self.set_key(self.ax)
        self.ax.text(self.keypos[0],self.keypos[1],"R2: {0}\ny={1}x +
{2}".format(Decimal(self.intDict[self.temp_choice][self.window][1])+Decimal(0),

Decimal(self.intDict[self.temp_choice][self.window][0])+Decimal(0),

Decimal(self.intDict[self.temp_choice][self.window][2])+Decimal(0)),
```

```
                color='k', fontsize = '10')


        if self.plot_type == 'single':
            self.ax2 = self.ax.twinx()
            self.logtime = False
            self.axis_assign()
            self.create_plot(self.ax2, colors=['g','y'])
            self.logtime = True
        if self.plot_type != 'single':
            self.ax.set_title(self.lnDF.columns[self.colnum])
            fig2 = plt.figure(figsize = (8.0,8.0))
            self.bx = fig2.add_subplot(111)
            self.bx.set_xlabel("Time(hours)")
            self.bx.set_ylabel(self.ylabels)
            if self.logtime == True:
                self.bx.plot(self.fullx,self.fully,'bo')
            else:
                self.bx.plot(self.fullx,self.fully,'go')
            self.bx.set_title(self.lnDF.columns[self.colnum])


            if self.logtime == False:

fig.savefig('{0}_OD_WINDOW.pdf'.format(self.save_file,self.lnDF.columns[self.colnum]),
format = 'pdf')

                fig2.savefig('{0}_OD.pdf'.format(self.save_file,self.lnDF.columns[self.colnum]),
format = 'pdf')
```

```
            self.logtime = True
            self.singleplot()
        else:

fig.savefig('{0}_LN_WINDOW.pdf'.format(self.save_file,self.lnDF.columns[self.colnum]),
format = 'pdf')
            fig2.savefig('{0}_LN.pdf'.format(self.save_file,self.lnDF.columns[self.colnum]),
format = 'pdf')




    def axis_assign(self):
        if self.plot_type == 'quad':
            if self.logtime == True:
                self.fully = self.lnDF[self.lnDF.columns[self.colnum]]
                self.fullx = self.lnDF.index
                self.yaxis = self.lnDF.ix[self.winList[self.window],self.colnum]
                self.ylimits =
[self.lnDF.loc[min(self.winList[self.window])][self.colnum],self.lnDF.loc[max(self.winList[s
elf.window])][self.colnum]]
                self.xlimits = [min(self.winList[self.window]),max(self.winList[self.window])]
                self.ylabels = 'Ln[OD]'
                return()
            else:
                self.fully = self.xx[self.xx.columns[self.colnum]]
                self.fullx = self.xx.index
                self.yaxis = self.xx.ix[self.winList[self.window],self.colnum]
```

```
            self.ylimits =
[self.xx.loc[min(self.winList[self.window])][self.colnum],self.xx.loc[max(self.winList[self.w
indow])][self.colnum]]

            self.xlimits = [min(self.winList[self.window]),max(self.winList[self.window])]

            self.ylabels = 'OD'

        else:

            if self.logtime == True:

                self.fully = self.lnDF[self.lnDF.columns[self.colnum]]

                self.fullx = self.lnDF.index

                self.yaxis = self.lnDF.ix[self.windowDict[self.temp_choice],self.colnum]

                self.ylimits =
[self.lnDF.loc[min(self.windowDict[self.temp_choice])][self.colnum],self.lnDF.loc[max(self.
windowDict[self.temp_choice])][self.colnum]]

                self.xlimits =
[min(self.windowDict[self.temp_choice]),max(self.windowDict[self.temp_choice])]

                self.ylabels = 'Ln[OD]'

            else:

                self.fully = self.xx[self.xx.columns[self.colnum]]

                self.fullx = self.xx.index

                self.yaxis = self.xx.ix[self.windowDict[self.temp_choice],self.colnum]

                self.ylimits =
[self.xx.loc[min(self.windowDict[self.temp_choice])][self.colnum],self.xx.loc[max(self.wind
owDict[self.temp_choice])][self.colnum]]

                self.xlimits =
[min(self.windowDict[self.temp_choice]),max(self.windowDict[self.temp_choice])]

                self.ylabels = 'OD'


    def set_key(self,axes):
```

```
    ymin, ymax = axes.get_ylim()
    xmin, xmax = axes.get_xlim()
    if self.plot_type == 'final':
        xkey = xmin + (xmax-xmin)/40
        ykey = ymax - (ymax-ymin)/10
    else:
        xkey = xmin + (xmax-xmin)/45
        ykey = ymax - (ymax-ymin)/7
    keys = [xkey,ykey]
    return(keys)

def create_plot(self,axes,colors=None):
    axes.set_xlabel('Time (h)')
    if colors != None:
        axes.set_ylabel(self.ylabels, color = colors[0])
        axes.plot(self.fullx, self.fully,'{0}o'.format(colors[0]))
        axes.plot(self.fullx, self.fully,'{0}-'.format(colors[0]))
        axes.plot(self.yaxis.index, self.yaxis,'{0}o'.format(colors[1]))
        axes.plot(self.yaxis.index, self.yaxis,'{0}-'.format(colors[1]))
    else:
        axes.set_ylabel(self.ylabels)
        if self.logtime == False:
            self.colorwheel = ['g','y']
        else:
            self.colorwheel = ['b','r']
```

```
        axes.plot(self.fullx, self.fully,'{0}o'.format(self.colorwheel[0]))
        axes.plot(self.fullx, self.fully,'{0}-'.format(self.colorwheel[0]))
        axes.plot(self.yaxis.index, self.yaxis,'{0}o'.format(self.colorwheel[1]))
        axes.plot(self.yaxis.index, self.yaxis,'{0}-'.format(self.colorwheel[1]))
    if colors == None and self.plot_type in ['quad','single']:
        if self.plot_type == 'quad':
            axes.set_title("Choice {0}".format(self.choiceDF.index[self.window]))
        elif self.plot_type == 'single':
            self.ax.set_title("Choice {0}".format(self.temp_choice))
    if colors != None:
        for tk in axes.get_yticklabels():
            tk.set_color(colors[0])
            tk.set_fontsize(10)
    elif self.plot_type in ['quad','single']:
        axes.set_ylabel(self.ylabels, color = 'b')
        for tk in axes.get_yticklabels():
            tk.set_color('b')
            tk.set_fontsize(10)
        for tk in axes.get_xticklabels():
            tk.set_fontsize(10)
    if self.keypos == None:
        self.keypos = self.set_key(self.ax)
    if self.logtime == True and self.plot_type == 'quad':
        xs,ys = self.fit_fn(self.yaxis.index, self.yaxis,2)
        axes.plot(xs,ys,'k-')
```

```
            axes.text(self.keypos[0],self.keypos[1],"R2: {0}\ny={1}x +
{2}".format(Decimal(self.intDict[self.choiceDF.index[self.window]][1][1])+Decimal(0),

Decimal(self.intDict[self.choiceDF.index[self.window]][1][0])+Decimal(0),

Decimal(self.intDict[self.choiceDF.index[self.window]][1][2])+Decimal(0)),
                    color='k', fontsize = '8')


    def quad_plot(self):
        getcontext().prec = 3
        self.index = 0
        self.window = 0
        self.axis_assign()
        self.fig = plt.figure(figsize=(8.0,8.0))



        self.ax = self.fig.add_subplot(221)
        self.create_plot(self.ax)
        self.ax2 = self.ax.twinx()
        self.logtime = False
        self.axis_assign()
        self.create_plot(self.ax2, colors=['g','y'])
        self.logtime = True
        self.window += 1
        self.axis_assign()
```

```
self.bx = self.fig.add_subplot(222)
self.create_plot(self.bx)
self.bx2 = self.bx.twinx()
self.logtime = False
self.axis_assign()
self.create_plot(self.bx2, colors=['g','y'])
self.logtime = True
self.window += 1
self.axis_assign()

self.cx = self.fig.add_subplot(223)
self.create_plot(self.cx)
self.cx2 = self.cx.twinx()
self.logtime = False
self.axis_assign()
self.create_plot(self.cx2, colors=['g','y'])
self.logtime = True
self.window += 1
self.axis_assign()

self.dx = self.fig.add_subplot(224)
self.create_plot(self.dx)
self.dx2 = self.dx.twinx()
self.logtime = False
self.axis_assign()
```

```
    self.create_plot(self.dx2, colors=['g','y'])
    self.logtime = True
    self.window += 1
    self.fig.tight_layout()



def fit_fn(self,x,y,i):
    coeff = np.polyfit(x,y,1)
    poly = np.poly1d(coeff)
    xs = []
    if self.logtime == False:
        for x in self.xx.index:
            if poly(x) <= max(self.xx[self.xx.columns[self.colnum]]):
                xs.append(x)
        ys = poly(xs)
        xs = np.array(xs)
        ys = np.array(ys)
        catch = [xs,ys]
    else:
        for x in self.lnDF.index:
            if poly(x) <= max(self.lnDF[self.lnDF.columns[self.colnum]]):
                xs.append(x)
        ys = poly(xs)
        xs = np.array(xs)
```

```
            ys = np.array(ys)
            catch = [xs,ys]
        return catch


    def __call__(self, pipe, plot_type, lnDF, winList, colnum, windowDict, temp_choice,
choiceDF, logtime, xx,intDict,save_file,bypass,examine):
        self.bypasslevel = bypass
        self.keypos = None
        self.save_file = save_file
        self.intDict = intDict
        self.logtime = logtime
        self.xx = xx
        self.choiceDF = choiceDF
        self.lnDF = lnDF
        self.winList = winList
        self.colnum = colnum
        self.plot_type = plot_type
        self.windowDict = windowDict
        self.temp_choice = temp_choice
        self.pipe = pipe
        self.gid = gobject.timeout_add(1000, self.poll_draw())
        if self.plot_type in ['single','final']:
            self.singleplot()
            if self.plot_type == 'final':
                plt.close('all')
```

```
        else:
            self.quad_plot()
            self.fig.savefig('{0}_Top4.pdf'.format(self.save_file,self.lnDF.columns[self.colnum]),
format = 'pdf')
        if self.bypasslevel[2] != True and self.bypasslevel[1] != True and self.bypasslevel[3] !=
True:
            if self.bypasslevel[0] == True and examine == False and self.plot_type in ['single']:
                pass
            elif self.plot_type in ['quad','single']:
                thismanager = get_current_fig_manager()
                thismanager.window.move(0, 0)
                plt.show()
            elif self.bypasslevel[0] == True and self.plot_type == 'quad':
                thismanager = get_current_fig_manager()
                thismanager.window.move(0, 0)
                plt.show()


if __name__ == '__main__':
    parser = argparse.ArgumentParser(description = "Calculate specific growth rate and lag
time for OD time series.")
    parser.add_argument("--quick", help='invoke to skip verification graphing and selection
step.',
                        action = 'store_true')
    parser.add_argument("--auto", help='invoke to override user selection & skip display of
graphs, will take top hit for all columns',
                        action = 'store_true')
```

```
    parser.add_argument("--noplot", help = "invoke to skip displaying graphs, graphs will still
be saved.",
                        action = 'store_true')
    parser.add_argument("--nosave", help = "invoke to skip saving & display of graphs, CSV
files will still be saved.",
                        action = 'store_true')
    parser.add_argument("-o", metavar = "OUTPUT", help = "Path to output folder where
files will be placed. WILL OVERRIDE files with the same name in the directory.")
    parser.add_argument("-winrange", help = "Minimum then Maximum window size,
separated by a space. Default = 6 15.",
                        nargs = 2, type = int, default = [6,15])
    parser.add_argument("-skiptocol", help = "Skip to a certain column in the datasheet.",
                        default = 0,type = int)
    parser.add_argument("--hardcap", help = "Cap all searches at a value.",
                        default = 0,type = int)
    args = parser.parse_args()
    dialog = gtk.FileChooserDialog("Select Input CSV File..",
                           None,
                           gtk.FILE_CHOOSER_ACTION_OPEN,
                           (gtk.STOCK_CANCEL, gtk.RESPONSE_CANCEL,
                            gtk.STOCK_OPEN, gtk.RESPONSE_OK))
    dialog.set_default_response(gtk.RESPONSE_OK)


    filter = gtk.FileFilter()
    filter.set_name("All files")
    filter.add_pattern("*")
    dialog.add_filter(filter)
```

```
    filter = gtk.FileFilter()
    filter.set_name("Images")
    filter.add_mime_type("image/png")
    filter.add_mime_type("image/jpeg")
    filter.add_mime_type("image/gif")
    filter.add_pattern("*.png")
    filter.add_pattern("*.jpg")
    filter.add_pattern("*.gif")
    filter.add_pattern("*.tif")
    filter.add_pattern("*.xpm")
    dialog.add_filter(filter)

    response = dialog.run()
    if response == gtk.RESPONSE_OK:
        input_file = dialog.get_filename()
    elif response == gtk.RESPONSE_CANCEL:
        raise ValueError('Closed, no files selected')
    dialog.destroy()
    print args.hardcap

spekit(args.o,input_file,args.winrange,args.quick,args.auto,args.noplot,args.nosave,args.skipt
ocol,args.hardcap)
    raw_input("Run completed with no errors. Press Enter to finish!")
```